MOTILE DAUGHTER COLONIES IN THE *CL. OEDEMATIENS* GROUP AND SOME OTHER CLOSTRIDIA (*CL. BOTULINUM C, CL. TETANI, CL. SEPTICUM*)

by A. W. TURNER AND CATHERINE E. EALES

(Animal Health Research Laboratory, Parkville, Victoria).

(Accepted for publication 28th April, 1941.)

INTRODUCTION.

The isolation and purification of motile micro-organisms by means of surface cultures is possible mainly because nearly all grow as more or less discrete and isolated colonies on the nutrient agar media commonly employed. Among those that do not form discrete colonies are the "swarmers," which are able to spread rapidly over the surface of agar and gelatin media through active migration of cell groups consisting of highly motile filamentous forms and behaving as small actively motile amoeboid daughter colonies.

Although *Proteus vulgaris* is apparently unique in being able to swarm on ordinary 1½ p.c. or 2 p.c. agar media when incubated aerobically at 37°C, many motile aerobes that under those conditions form discrete colonies can be induced to swarm by lowering the proportion of agar (Gard, 1938) or by adding gelatine to it (Dimitrijevic-Speth, 1929; Ivanic and Dimitrijevic-Speth, 1931). Several of the motile anaerobic sporulating bacilli (clostridia) swarm very readily even on ordinary blood agar or serum agar.

Distinct from swarming as a method of spreading is the phenomenon of motile colonies, first observed by Muto (1940) when studying an undetermined bacterium and since described in a group of terminal-spored aerobic bacilli by various workers (see Turner and Eales, 1941). These bacilli form colonies, of microscopic or often macroscopic dimensions, which glide *en masse*, usually in spiral paths, over the surface of agar media that are not sufficiently moist to support ordinary swarming.

So far as we are aware, this type of migration has not hitherto been recognized among the clostridia. Over a period of years we have observed, at first among members of the *Cl. oedematiens* group and subsequently among some other clostridia, a similar type of migration, but differing from it mainly in that large individual colonies have never been observed to move. Migration has been observed only in relatively small cell groups. These detach themselves from the mother colonies or from the edge of a swarm film, wander away on more or less spiral paths, often of great complexity, and behave virtually as motile daughter colonies.

Since most of our observations were made upon the *Cl. oedematiens* group, the main emphasis of this communication is upon them.

Nomenclature: Cl. oedematiens Group. The term "*Cl. oedematiens (Cl. novyi) Group*" is used in its widest sense to include not only the *Cl. oedematiens* of Weinberg and Séguin but certain closely related clostridia (Weinberg, Nativelle and Prévot, 1937). The provisional classification suggested by Scott, Turner and Vawter (1933) is used, according to which the species *Cl. oedematiens* is subdivided into three types as follows:

Type A: The "classical" medium-sized glycerin-fermenting *B. oedematiens* of Weinberg and Séguin, *Cl. novyi* of American authors or *Novybazillus* of German authors.

Type B: The distinctly larger, non-glycerin-fermenting black-disease bacillus of sheep (Turner, 1930) and the closely related *B. gigas* isolated by Zeissler and Rassfeld (1929) from bradsot of sheep in Germany.

Type C: The bacillus of *osteomyelitis bacillosa bubalorum* isolated by Kraneveld (1930) from cases of bacillary osteomyelitis of buffaloes in the Dutch East Indies and claimed by Zeissler and and him (1929) to be closely related to *B. gigas*.

We have carried out further observations, including "H" and "O" antigenic analysis, which confirm the close relationship of these species and support their classification as "types" of the species *Cl. oedematiens.*

TECHNIQUE.

The observations were made under the plate culture microscope usually upon cultures on 2 p.c. V.F. agar[1] containing 10 p.c. of sterile defibrinated sheep blood. Before use, the plates were incubated overnight at 37° C. to control sterility and to evaporate excessive water, and were stored at 3° C. They were quite free of perceptible surface moisture. Plates were usually incubated anaerobically at 37° C. by Fortner's method (1928). Less often, plates were incubated anaerobically in the McIntosh and Fildes jar, or other media were used. Impression preparations were made by Bissett's technique (1938).

OBSERVATIONS.

(a) *Cl. oedematiens group.* Under our conditions of cultivation, the appearances to be described are not always seen with regularity: sometimes several plates may have to be examined before the phenomenon occurs. Apparently, certain surface conditions are necessary and are more critical with some strains than with others. We have not been able to define the conditions accurately. With "difficult" strains, it may be confined to colonies near the wall of the Petrie dish. It occurs always with Type C; in other strains in which it has been observed, it occurs more regularly with Type B than with Type A.

Thirty-six strains were studied and the phenomenon was observed in 5 out of 9 (55 p.c.) classed as Type A, 13 out of 22 (59 p.c.) classed at Type B, and 6 out of 6 (100 p.c.) classed as Type C.

Since many of the strains studied may be in other collections, the information concerning them is collected in Table 1.

TABLE 1.

Strains of Cl. oedematiens *group examined.*

Type.	Strain.	Source.	Host.	Motile Colonies.
A	Cossard, N.C.T.C. 277	National Collection of Type Cultures, London (N.C.T.C.)	Man	+
	Zeissler	Prof. J. Zeissler, Germany	Not known	+
	140, N.C.T.C. 2908	N.C.T.C., ex Prof. Ivan Hall, ex Prof. Weinberg	Man	+
	Lister	Commonwealth Serum Labs., Melbourne	Man	+
	Jolly, N.C.T.C. 538	N.C.T.C.	Man	+
	Domange, N.C.T.C. 278	N.C.T.C.	Man	—
	Weinberg	Prof. Weinberg, Paris	Man	—
	Hall 139	Prof. Ivan Hall, U.S.A.	Horse	—
	Collins	Dr. L. B. Bull, Melbourne	Sheep, South Australia, "Big Head"	—
B	B.D. Tongala	Own isolation	Sheep, Victoria, Black Disease	+
	B.D. 24	" "	Ditto	+
	L.L. 47	" "	Ditto	+
	B.D. Oxer	Mr. D. T. Oxer, Melbourne	Sheep, Tasmania, Black Disease	+
	B.D. Rose 1	Mr. G. Edgar, Glenfield, N.S.W.	Sheep, N.S.W., Black Disease	+
	B.D. Rose 3	" " " " "	Ditto	+
	Hopkirk	Dr. C. S. M. Hopkirk, New Zealand	Sheep, New Zealand, Black Disease	+
	S.B. 42	Mr. J. A. Dumaresq, Tasmania	Horse, Tasmania, Black Disease	+

[1] V.F. (= "viande-foie") is a pig-stomach digest of ox muscle and ox liver, as used in Weinberg's laboratory at the Pasteur Inst., Paris (see Turner, 1930).

TABLE 1 (continued)

Type.	Strain.	Source.	Host.	Motile Colonies.
	Gigas 684	Prof. J. Zeissler, Hanover, Germany	Sheep, Germany, Bradsot	+
	Gigas 1078	" " " "	Ditto	+
	Gigas 1211	" " " "	Ditto	+
	Gigas 1351	" " " "	Ditto	+
	S.N. 737, N.C.T.C. 3264	N.C.T.C., ex Dr. H. R. Seddon, Glenfield	Sheep, N.S.W., Black Disease	+
	B.D. Albiston	Dr. H. E. Albiston, Melbourne	Sheep, Victoria, Black Disease	—
	B.D. Rose 2	Mr. G. Edgar, Glenfield	Sheep, N.S.W., Black Disease	—
	B.D. 10	Own isolation	Sheep, Victoria, Black Disease	—
	B.D. 19	" "	Ditto	—
	B.D. 58	" "	Ditto	—
	R.B. 977	" "	Cow, Victoria, Black Disease	—
	Gigas 6	Prof. J. Zeissler	Sheep, Germany, Bradsot	—
	Gigas 12 A	" " "	Ditto	—
C	O.B.B. 1	Dr. F. C. Kraneveld, Java	Water Buffalo, Java. Osteomyelitis bubalorum bacillosa	+
	O.B.B. 2	" " " " "	Ditto	+
	O.B.B. 3	" " " " "	Ditto	+
	O.B.B. 4	" " " " "	Ditto	+
	O.B.B. 5	" " " " "	Ditto	+
	O.B.B. 6	" " " " "	Ditto	+

The following appearances are typically seen. From the periphery of the irregular opaque contoured colonies groups of cells, comprising from a few hundreds to a few thousands, detach themselves and, behaving as minute daughter colonies usually from 0·1 to 0·3 mm. in diameter, move away from the mother colony. The cells align themselves parallel to the direction of movement and the daughter colonies assume elongated rather tongue-shaped (Fig. 1, Nos. 1 and 2) or heart-shaped masses (Nos. 3, 4, 5, 6 and 7) with convex anterior borders. These glide as units over the surface of the medium, usually pursuing more or less regular spiral paths which may be clockwise, anticlockwise or may begin in one direction and change to the other. They may take quite convoluted paths, crossing and recrossing the original path (No. 8). Most show a tendency to return eventually towards the mother colony and may then fuse with it. As they migrate, they lightly smear the surface of the agar leaving tracks which resemble the slimy tracks left by snails. A certain number of bacilli are cast off (No. 12), mostly from the edges of the moving colony, are left behind and may later multiply; a peculiar track, in the form of two parallel lines is thus formed wherever the daughter colony has wandered. The tracks may reach a centimetre or even 3 cm. in length (No. 8), and are the same width as the actively migrating colonies. With Type A, the wandering daughter colonies are usually small and consequently the "tracks" left by them are usually narrow (up to 0·15 mm.). The same applies to most strains of Type B, but with some of the "gigas" strains and the Type C strains they may be relatively large (up to 0·37 mm.). After a certain period of migration the gliding daughter colony may come to rest, or the spiral path may acquire a rapidly decreasing radius before the colony finally comes to rest. In the resting stage, the satellite daughter colony may increase in size up to 5 or 6 times its original diameter (i.e. usually up to about 0·5 mm.) and assume the irregular, contoured appearance of the mother colony, but on a smaller scale.

Impression preparations of young migrating daughter colonies indicate that in Type-A strains the cells are only slightly longer than in the main colony. They are aligned in the direction of colony movement and those that are left behind during movement tend to stain lighter. In Type-B and Type-C strains, in which the tendency to filament production is greater, the leading

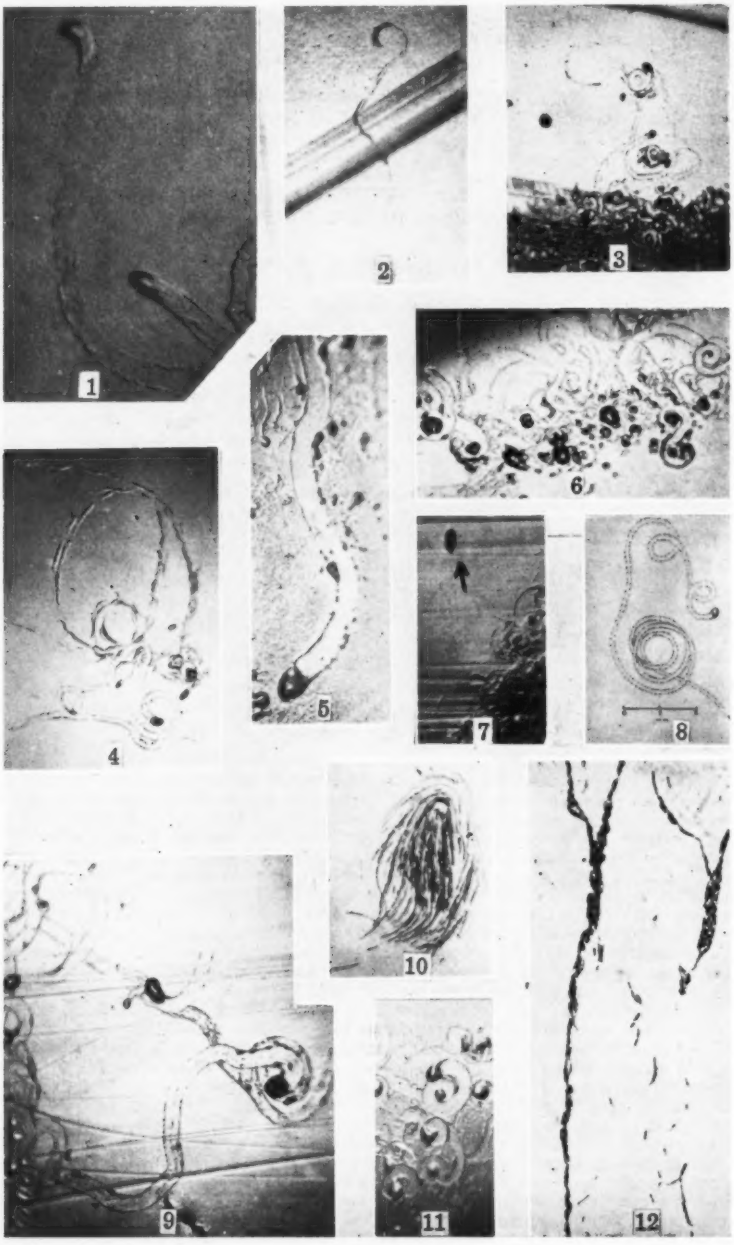


Figure 1.

edge of the colony is often sheathed by long filamentous forms with their ends streaming backwards (No. 10). The track consists of two parallel lines of irregularly discarded shorter rods (No. 12) similar to those that constitute most of the mother colony. This morphological differentiation between the actively motile and the resting forms recalls that between the filamentous swarming rods and the shorter resting forms of swarming bacteria. Smears from the moving colonies and the track show the long filamentous forms richly endowed with luxuriant flagella whereas the discarded shorter forms have fewer flagella.

Some Type-B strains and all Type-C strains swarm very readily under our conditions and thus discrete colonies are not easily obtained. In such cases, motile cell-groups may invade the medium ahead of the swarm film and are then similar in behaviour to the daughter colonies described above. Similar motile cell groups also arise within the swarm film and sweep their way through it, leaving relatively bacteria-free paths with heaped edges (Fig. 1, Nos. 5, 11), as described for our aerobic bacillus (Turner and Eales, 1941).

The appearances described by us in cultures of our aerobic bacillus are thus nearly all paralleled by similar appearances in cultures of *Cl. oedematiens*. They are not so obvious, because they may be on a smaller scale and may need the assistance of optical magnification and suitable lighting to be readily appreciated.

(b) *Wandering daughter colonies among other clostridia.* We have examined many hundreds of plate cultures of 65 strains of members of the motile clostridia, and have never observed motile cell groups, as described above for *Cl. oedematiens*, in any strains of the following species: *Cl. haemolyticum* (closely related antigenically and toxinogenically with some members of the *Cl. oedematiens* group), 3 strains; *Cl. tetanomorphum*, 4 strains; *Cl. putrificum*, 2 strains; *Cl. tertium*, 5 strains; *Cl. cochlearium*, 1 strain; *Cl. sphenoides*, 1 strain; *Cl. sporogenes*, 8 strains; *Cl. parasporogenes*, 1 strain; *Cl. centrosporogenes*, 1 strain; *Cl. bifermentans*, 6 strains; *Cl. multifermentans*, 1 strain; *Cl. multifermentans-tenalbus*, 1 strain; *Cl. aerofoetidum*, 1 strain; *Cl. fallax*, 4 strains; *Cl. histolyticum*, 2 strains; *Cl. chauvoei*, 17 strains; *Cl. botulinum* Type A, 2 strains; *Cl. botulinum* Type B, 2 strains; and *Cl. botulinum* Type D, 2 strains.

We have observed motile daughter colonies occasionally in the following species: one strain of *Cl. botulinum C* "178 C" from the Veterinary Research Laboratories, Onderstepoort, South Africa (Fig. 2, No. 13), two strains of *Cl. botulinum* Cβ (= *Cl. parabotulinum* Seddon) from Professor H. R. Seddon and from the N.C.T.C. respectively, two strains of *Cl. tetani* "N.C.T.C. 279" and "Pease" respectively (Nos. 14, 15, 16), one strain of *Cl. septicum* isolated from a sheep (No. 17), and an undetermined terminal-spored clostridium "205 Cr" isolated from a sheep. In all except the last, the phenomenon was associated with swarming and the motile cell groups or colonies pursued their spiral paths ahead of or amongst the swarm film; in the last (No. 18) it resembled closely the appearances seen in *Cl. oedematiens* when not swarming.

(c) *Swarming among clostridia.* Swarming in *Proteus* is essentially a continuous or rhythmical centripetal invasion of the whole surface of the medium by very small groups of cells, ever changing in shape, sometimes breaking into smaller groups or fusing temporarily with other

---

EXPLANATION OF FIGURE 1.

No. 1. *Cl. oedematiens* Type A "Jolly", 24 hrs., showing tracks and moving colonies. × 40.

No. 2. *Cl. oedematiens* Type B "L.L. 47", 48 hrs., showing elongated wandering daughter colony at end of a track. × 10.

No. 3. *Cl. oedematiens* Type B "Gigas 684", 48 hrs., showing several elaborate tracks formed by wandering daughter colonies. × 10.

No. 4. *Cl. oedematiens* Type B "B.D. Tongala", 24 hrs. The discarded bacilli along the tracks, especially the upper one, have multiplied considerably. × 10.

No. 5. *Cl. oedematiens* Type B "Gigas 1211", 24 hrs., showing a large wandering cell group or daughter colony and several smaller ones pushing their way through the swarm film. Note the heaped-up, relatively bacteria-free track. × 10.

No. 6. *Cl. oedematiens* Type B "B.D. Oxer", 24 hrs., showing numerous wandering daughter colonies and fairly short tracks. × 10.

No. 7. *Cl. oedematiens* Type A "Zeissler", 24 hrs., McIntosh and Fildes' jar, showing tracks at edge of a colony and a small wandering daughter colony (indicated by arrow). × 10.

No. 8. *Cl. oedematiens* Type A "Jolly", 24 hrs. Sketch of a moving colony with a very long (about 3 cm.) and elaborate track.

No. 9. *Cl. oedematiens* Type B "Gigas 1211', 72 hrs., showing large daughter colonies and well-defined tracks. × 10.

No. 10. *Cl. oedematiens* Type B "Gigas 1078", 24 hrs., impression preparation of moving colony showing how its anterior edge is sheathed with long filaments. × 120.

No. 11. *Cl. oedematiens* Type B "BD 24", 24 hrs., showing numerous wandering cell groups sweeping clean curved tracks through swarm film. × 10.

No. 12. *Cl. oedematiens* Type B "S.B. 42", 24 hrs., impression preparation of track of a moving colony, showing that the edge of the track is composed of discarded cells. The tracks of two colonies have coincided, but diverge above. The right lower part of the common track has been badly distorted in making the impression preparations.

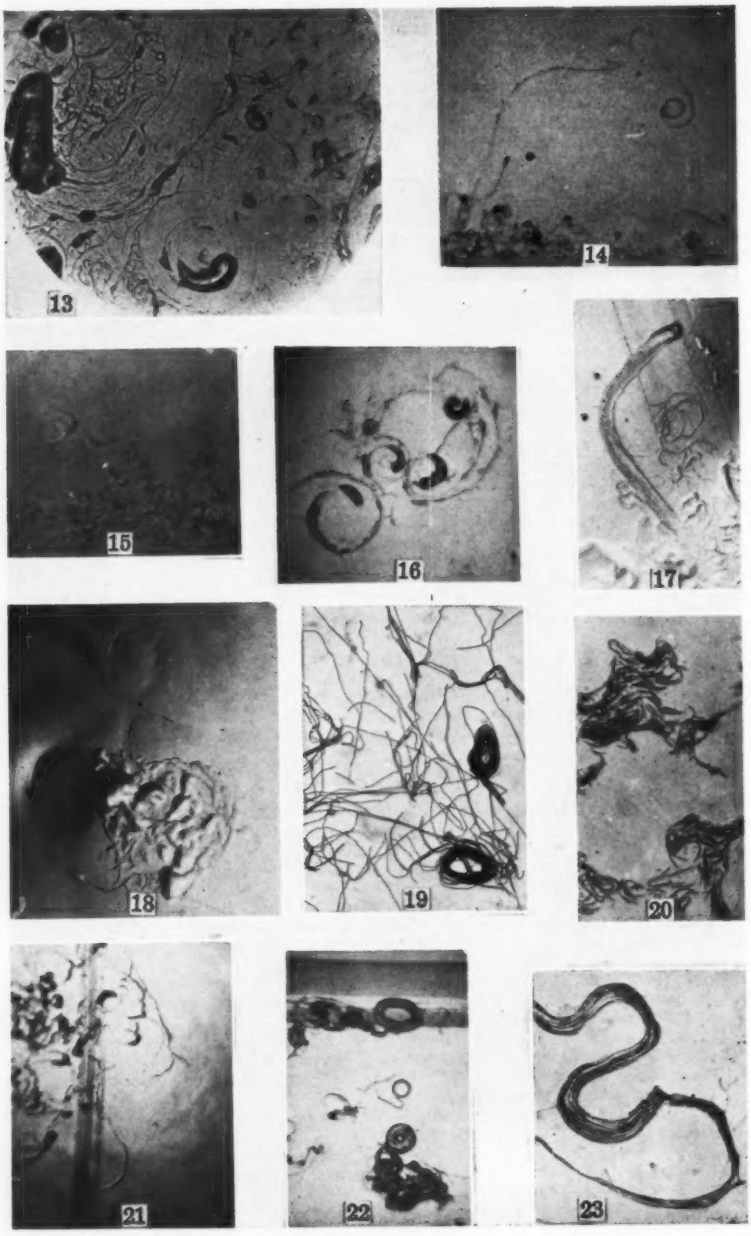


Figure 2.

groups. In the clostridia the swarming groups more usually form great fusiform tresses of long filaments, as in *Cl. septicum* (Nos. 21 and 23). In *Cl. oedematiens* Type C (i.e. O.B.B. bacillus) and in *Cl. botulinum* C and Cβ, they consist of fusiform tresses of long filaments, together with enormous individual filaments and parallel bundles of filaments which coil up to form peculiar annular satellite colonies (Nos. 19 and 22) and are later incorporated in the swarm film.

DISCUSSION.

Through technical difficulties we could not study the cultures anaerobically and therefore have not observed the daughter colonies actually migrating. Nevertheless, all the appearance so closely resemble those described for certain aerobic bacilli (see Turner and Eales, 1941) that there is no doubt that our explanation is correct.

From published descriptions of the *Cl. oedematiens* group it is obvious that the German school, influenced by Zeissler, regard many of the appearances as characteristic, constant and of great determinative value (Zeissler, 1930; Kraneveld, 1930; Miessner, Meyn and Schoop, 1931; Jansen, 1932, 1934.) We believe, however, that they have interpreted their observations wrongly. What they describe as "characteristic, double parallel filamentous outgrowths which frequently return into or towards the colony" are the tracks of discarded bacilli left by the wandering daughter colonies. What they describe as "small massive knots (or nodes) at the ends of the outgrowths" are the wandering daughter colonies themselves at various stages of development.

We observed the phenomenon in only about half of our "classical" (i.e. Type A) strains and then sometimes only after repeated trials. Its occurrence among certain other clostridia, including *Cl. botulinum*, *Cl. tetani* and *Cl. septicum*, indicates that under suitable conditions many motile bacteria may exhibit it.

It is curious that French, British and American workers have not recorded the appearances. There are two probable reasons: the media and cultural conditions employed by Zeissler and the insistence he places upon the use of the plate culture microscope.

The biological value of migrating colonies has been discussed by Turner and Eales (1941). The phenomenon is distinct from swarming, although sometimes associated with it. The cell groups or daughter colonies are organized in a more orderly fashion. They pursue peculiar curved spiral or circular paths and may return to the mother colony and fuse with it. The phenomenon may and often does occur under conditions unsuitable for swarming.

Knöll (1940) showed that the rhythmical swarming of *Pr. vulgaris* is not due to hunger and over-population as postulated by Russ-Münzer (1935), but

EXPLANATION OF FIGURE 2.

No. 13. *Cl. botulinum* C "178 C", 24 hr. cult. on blood agar showing numerous motile cell groups pursuing spiral or circular paths in the swarm film. × 10.

Nos. 14–16. *Cl. tetani* "Pease", 24 hr. cult. on blood agar, showing motile daughter colonies and tracks left by them. × 10.

No. 17. *Cl. septicum* "15 P.F.", 24 hr. cult. on blood agar, showing a motile cell group that has left a track on which discarded cells have subsequently mutiplied. × 10.

No. 18. *Undetermined terminal-spored clostridium*, 20 hr. cult. on blood agar, showing one motile daughter colony near mother colony, and tracks left by others. × 10.

No. 19. *Cl. oedematiens* Type C "O.B.B. 3", 24 hr. cult. on blood agar. Impression preparation at edge of swarm film showing long filaments and annular masses of coiled filaments. × 10.

No. 20. *Proteus vulgaris*. Impression preparation, 5½ hr. culture on agar, showing amoeboid swarming groups at edge of swarm film. × 10.

No. 21. *Cl. septicum* "15 P.F.", 17 hr. cult. on blood agar, showing edge of swarm film. × 10.

No. 22. *Cl. oedematiens* Type C "OBB 3". Culture from which No. 19 was made. × 10.

No. 23. Impression preparation from above culture, showing enormous tresses of filaments. × 10.

to moisture and food fluctuations through the colloidal medium, the whole process being akin to Liesegang's rings. It is probable that the migration of colonies and cell-groups is similarly due to physico-chemical forces such as diffusion of nutrients and osmotic attraction of water by metabolic products. The complex and intriguing paths, which tempt teleological explanations, may be partly the result of preformed colloidal surface differences.

So far as the clostridia are concerned, it is difficult to imagine a set of circumstances in nature, combining a nutrient surface with the optimal water concentration and an anaerobic atmosphere, that would give the members of this genus the opportunity of profiting from their capacity to spread by means of motile daughter colonies. As a matter of practical bacteriology, however, the phenomenon is important as a possible source of difficulty in obtaining pure cultures. Obviously, wandering daughter colonies that return towards or into the mother colony may easily introduce contamination from neighbouring colonies. It emphasizes the necessity in all surface culture technique of using a low power lens, or, better, the plate culture microscope.

SUMMARY.

Twenty-four out of 37 strains, representing the three types of the *Cl. oedematiens* group distinguished by Scott, Turner and Vawter (1930) were found capable of giving rise to wandering daughter colonies on the surface of agar media.

The phenomenon is essentially similar to that observed in certain aerobic terminal-spored bacilli.

It was also observed in some strains of *Cl. botulinum* Type C, *Cl. tetani* and *Cl. septicum* and an undetermined terminal-spored anaerobe.

Although some of these appearances have been described before in members of the *Cl. oedematiens* group they have hitherto been misinterpreted.

The relationship of this phenomenon to swarming is discussed.

REFERENCES.

Bissett, K. A. (1938): J. Path. Bact., 47, p. 223.
Dimitrijevic-Speth, V. (1929): Zbl. Bakt. I Orig., 113, p. 524.
Fortner, J. (1928): *Ibid.*, 108, p. 155.
Gard, S. (1938): Z. Hyg., 120, p. 615.
Ivanic, S. Z. and Dimitrijevic-Speth, V. (1931): Zbl. Bakt. I Orig., 119, p. 271.
Jansen, J. (1932): Tijd. Diergeneesk, 59, p. 174.
Jansen, J. (1934): *Ibid.*, 61, p. 638.
Knöll, H. (1940): Kolloid-Z., 89, p. 135.
Kraneveld, F. C. (1930): Ned.-Ind. Bl. Diergeneesk, 42, p. 564.
Miessner, H., Meyn, A. and Schoop, G. (1931): Zbl. Bakt. I Orig., 120, p. 257.
Muto, T. (1904): *Ibid.*, 37, p. 321.
Russ-Münzer, A. (1935): *Ibid.*, 133, p. 214.
Scott, J. P., Turner, A. W. and Vawter, L. R. (1933): Proc. 12th intern. Vet. Congr., New York, p. 168.
Turner, A. W. (1930): Bull. Coun. sci. indust. Res., Austral., No. 46.
Turner, A. W. and Eales, C. E. (1941): Austral. J. exp. Biol., 19, p. 161.
Weinberg, M., Nativelle, R. and Prévot, A. (1937): "Les microbes anaérobies". Masson et Cie: Paris, p. 259.
Zeissler, J. (1930): "Anaërobenzüchtung", in Kolle, Kraus and Uhlenhuths Handbuch d. pathog. Mikroorganismen, 10, p. 108.
Zeissler, J. and Kraneveld, F. C. (1929): Arch. wiss. prakt. Tierhlk., 60, p. 441.
Zeissler, J. and Rassfeld, L. (1929): *Ibid.*, 59, p. 419.

THE LIBERATION OF PHARMACOLOGICALLY ACTIVE SUBSTANCES FROM TISSUES BY CHLOROFORM

by E. R. TRETHEWIE

(From the Department of Physiology, University of Melbourne).

(Accepted for publication 21st May, 1941.)

INTRODUCTION.

It has been shown that when the concentration of chloroform in the air ventilating the isolated lung perfused with Tyrode rises to 4·4 p.c., histamine and a slow-reacting smooth-muscle stimulating substance (S.R.S.) appear in the perfusate (Kellaway and Trethewie (1939)). The present paper concerns injury by chloroform to the isolated perfused and intact liver of the dog and the isolated perfused head of the guinea-pig.

Investigations have been made upon the liberation of adenyl compounds and of an inactivating enzyme from the liver as has been shown in injury by cobra venom (Kellaway and Trethewie (1940)), and the liberation of a substance causing contraction of the dorsal muscle of the leech and frog rectus from the perfused head of the guinea-pig. In addition, the liberation of histamine and S.R.S. have been investigated.

METHODS.

Perfusion experiments. Dogs were anaesthetized with morphia (2 grains subcutaneously), lightly chloroformed, and the thorax rapidly opened through the diaphragm from the abdomen. The liver was perfused via the portal vein with Tyrode solution at a rate of 1·5–2·5 c.c. per minute as described by Feldberg and Kellaway (1937). The perfusate was collected over a boiling water bath before and for periods 0–3, 3–10, 10–30 and 30–60 minutes after the injection of chloroform except when specifically stated. Injections of chloroform were made directly into the intraportal cannula and the flow stopped for one minute. When doses of 0·1 c.c. and less were used the chloroform was first emulsified in 1 c.c. of serum (obtained from the dog before excision of the liver) and then injected. Extracts of liver were made by grinding the organ with silica in Tyrode and then boiling.

Guinea-pigs were anaesthetized by the subcutaneous injection of 2·5 gm. urethane. The head was perfused via cannulae in both carotids with Locke's solution to which eserine was added to make a concentration of 1 in 80,000. The jugular veins on each side were divided, both internal and external, and the thorax was cut across just below the axillae. A cervical ligature was tied immediately cephalic to the fore-limbs which were amputated. The spinal canal was stopped with wooden chips and the aorta ligated about the arch. When the outflowing perfusate became clear of blood a sample was collected in the cold at a rate of 2 c.c. per minute. Injections of chloroform (0·05–0·1 c.c.) were made into one or both carotid cannulae, the flow was interrupted for one minute, and the second sample was collected in the cold for one hour after re-establishing the flow. Extracts of brain were made by grinding the cerebrum with silica in eserinized (1 in 20,000) diluted Locke's solution (35 c.c. water added to 100 c.c. Locke's solution) and boiling.

Assay of perfusate and extracts of organs. Tests for adenyl compounds were made on the guinea-pig heart preparation (Drury, Lutwak-Mann and Solandt (1938)).

Acetylcholine was estimated on the anterior part of the eserinized leech muscle (Minz (1932), Feldberg and Krayer (1933)), and on the frog rectus abdominis (Chang and Gaddum (1933)). Tests were also made on the isolated guinea-pig jejunum suspended in a 7 c.c. bath of Tyrode solution before and after atropinization, and similarly upon the carotid blood pressure of the cat anaesthetized with ether and chloralose, the vagi being divided.

Histamine and S.R.S. were estimated upon the isolated guinea-pig jejunum, and the former also upon the blood pressure of the cat as above. Acetylcholine was estimated as the hydrochloride, adenyl compounds as adenosine, and histamine as histamine phosphate.

RESULTS.

Experiments on Dogs.

(a) *The liberation of adenylic acid from the isolated perfused liver.* The liver is an organ rich in adenylic acid (Bennett and Drury (1931)) and in the dog

the normal content is equivalent to 0·17–0·9 mg. adenosine per gm. This organ can be perfused with Tyrode solution for 1½ hours without the appearance of any adenyl compound in the perfusate and without any significant loss of adenyl compound from the organ as determined by comparison of the perfused lobe with other portions of the excised liver. Perfusion of the liver with Tyrode solution for half an hour followed by the intraportal injection of 1 c.c. of the dog's serum and at the same time interrupting the flow for one minute, followed by further perfusion for one hour is also without effect. The injection of chloroform into the portal vein of the perfused liver of the dog causes an immediate output of adenyl compounds (Table 1) and also of an agent capable of inactivating them. With small doses (0·07 to 0·15 c.c. chloroform to approximately 80 gm. tissue) the output was maximal in 3–10 minutes following injection, but with large doses (0·2 to 1 c.c.) the maximal output occurred in the first three minute sample after injection. In association with this effect with the smaller doses reticulation of the organ produced by depigmentation at the periphery of the lobules occurred gradually from 2–3 minutes after injection, whereas with the large doses the reticulation was immediate and depigmentation of the organ continued throughout the perfusion. The pigment was observed to pass into the perfusate, which also readily frothed on shaking. An output of protein coagulable by heat was also observed and this was greater following the injection of bigger doses.

TABLE 1.

Liberation of adenyl compounds by chloroform from the perfused liver.

Concentration $CHCl_3$ injected p.c. v/w.	Loss adenyl compound as p.c. by difference organ extracts.	Output in perfusate as mg. adenosine.	Output as p.c. of organ content.
*0·08	24	0·10	0·15
0·10	48	0·08	0·4
0·11	43	0·36	0·45
0·3	67	0·22	1·0
1·9	38	3·42	16

* Perfused for 25 minutes.

These findings are illustrated in the following experiment. A piece of liver weighing 111·3 gm. was perfused via the portal vein with Tyrode solution at a rate of 2·5 p.c. per minute and the perfusate collected at 95° C. was free of cardio-depressant activity. After 17 minutes an injection of 0·12 c.c. of chloroform emulsified in 1 c.c. of serum obtained from the same dog was made and the flow stopped for one minute (concentration 0·11 p.c.). On re-establishing the flow the rate of perfusion, the pressure remaining the same, was found to have fallen to 1·7 c.c. per minute and the rate continued to decline throughout the experiment. The first sample collected 0–3 minutes after injection (5 c.c.) contained cardio-depressant activity equivalent to 3γ adenosine per c.c. when tested on the guinea-pig heart preparation. The second sample obtained 3–10 minutes (11 c.c.), and the third obtained 10–30 minutes (21 c.c.) and the fourth obtained from 30 minutes to 1 hour after injection (34 c.c.) contained adenyl compounds equivalent to 9γ, 3·5γ and 5γ adenosine per c.c. respectively. Chloroform added to Tyrode (0·12 c.c. to 5 c.c.) and boiled was without effect in a dose of 1 c.c. The first sample following injection was very turbid, due to the presence of coagulated protein, and this was increased in the second, diminished in the third, and again increased in the fourth sample. There was no coagulable protein present in the sample obtained before injection. A control extract of liver contained adenyl compounds equivalent to 0·7 mg. adenosine per gm. The perfused liver after the experiment, which weighed 110·9 gm., contained adenyl compounds equivalent to 0·4 mg. adenosine per gm.

In another experiment where 0·15 c.c. chloroform was injected into a liver weighing 49·9 gm. the samples following injection contained adenyl compounds equivalent to 2γ adenosine per c.c. (3γ in all), 5·5γ per c.c. (53γ), 2γ per c.c. (86γ) and 1·5γ per c.c. (77γ) and the liver after the experiment contained the equivalent of 0·21 mg. adenosine per gm., whereas a control sample contained the equivalent of 0·7 mg. adenosine per gm. In Fig. 1 are shown the responses at 1, 2, 3, 4 and 5 to 1 c.c. of the samples: 1, before injection, and 2, 3, 4 and 5 the successive samples 0–3, 3–10, 10–30 and 30–60 minutes after injection. At A_1, A_2, A_3, A_5 and A_7, are shown the responses to 1γ, 2γ, 3γ, 5γ and 7γ adenosine respectively. Chloroform added to Tyrode in the proportion 0·1 c.c. to 1 c.c. and boiled (the concentration in sample 2) was without effect.

Histamine is also present in the liver of the dog in appreciable amounts, but in these experiments it did not interfere with the titration of adenyl compounds. The histamine content of extracts and samples of perfusate was estimated by the contraction of the guinea-pig jejunum and also checked by depression of the blood pressure of the atropinized cat, and the injection of amounts of histamine so estimated to be present in these samples were found alone to produce no cardiac depression when tested on the guinea-pig heart preparation. Histamine in a concentration of 1: 800,000 in mixtures with adenosine (1 in 100,000) is without effect in a dose of 1 c.c. on the depth and width of the cardiac depression as seen on the tracing compared with that produced by adenosine alone. With concentrations of histamine 1 in 500,000 and 1 in 350,000 there is a definite reduction in the response to adenosine. In one experiment histamine in a concentration of 1 in 350,000 caused an apparent reduction in response by 10 p.c. of adenosine (1 in 70,000) in a dose of 0·7 c.c. In these experiments where histamine was present in perfusate in a concentration greater than 1 in 800,000 it was diluted before testing.

(b) *The liberation of inactivating enzyme from the isolated perfused liver.* In Table 1 are shown the outputs of adenyl compounds following the injection of chloroform. These varied from 0·15–16 p.c. of the content of the lobe injected. The loss from the organ however varied from 24–67 p.c. This discrepancy is to be explained by the liberation of an enzyme capable of inactivating adenyl compounds as has been shown for cobra venom (Kellaway and Trethewie (1940)). This was investigated both by collecting the perfusate at 20°C, and also by allowing the outflowing drops to fall over a knife edge, one part passing to a flask at 20°C and the other to a water bath at 95°C. To the unheated perfusate adenosine was added (1 : 50,000–1 : 100,000) and portions removed at varying times following incubation, immediately boiled, and then tested on the atropinized guinea-pig heart preparation.

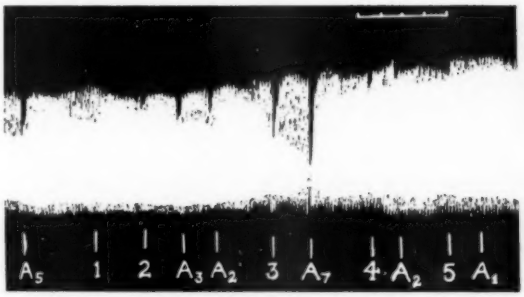


Fig. 1. Responses of the right auricle of the atropinized guinea-pig heart to perfusate from a perfused dog liver into which 0·15 c.c. chloroform was injected. Time in half minutes. Details in text.

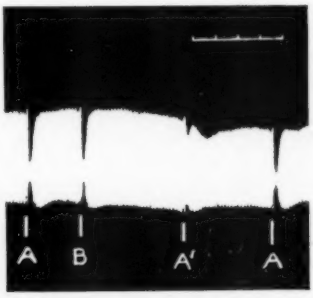


Fig. 2. Responses of the right auricle of the atropinized guinea-pig heart to unboiled perfusate to which adenosine was added, obtained from a perfused liver of a dog before and after the injection of chloroform. Time in half-minutes. Details in text.

In one experiment 0·2 c.c. of chloroform was injected into a piece of liver weighing 76 gm. (a concentration of 0·25 p.c.). Adenosine was added to perfusate collected before injection (32 c.c. in 21 minutes), and after injection (44 c.c. in 30 minutes) to make a concentration of 1: 50,000. At the end of 15 minutes incubation at 38° C. only 40 p.c. of the activity remained in the sample collected after injection and in 30 minutes only 20 p.c. of activity remained. The sample obtained before injection still contained activity equivalent to adenosine 1: 50,000 at the end of 30 minutes. In Fig. 2 are shown the responses at A to 0·5 c.c. of the sample obtained after injection to which adenosine was added and immediately boiled, at A^1 to 0·5 c.c. of the same sample, adenosine added, incubated for fifteen minutes and then boiled, and at B to 0·5 c.c. of the sample obtained before injection, adenosine added, and incubated for 15 minutes before boiling. If this result be graphed it can be computed that 100 c.c. of such perfusate was able to break down 1 mg. of adenosine in approximately ten minutes under these conditions. Since it

appeared that the loss by enzyme action was far greater than the amount of adenosine measured in the perfusate it was thought that it might be that the enzyme was liberated at lower concentrations of chloroform than was adenylic acid. If a dose could be selected where no adenyl compound could be detected in perfusate collected at 95° C., remembering that the time of passage of perfusion fluid in the vessels of the liver is very short, yet still much enzyme activity could be detected, then the result would favour such an hypothesis. Now with a concentration of 0·10 p.c. chloroform adenyl compounds were just detectable in the perfusate (equivalent to 1γ per c.c.) (Table 1). Therefore a dose of 0·07 c.c. chloroform emulsified in 1 c.c. of the dog's serum was injected intraportally into a liver weighing 92 gm. (a concentration of 0·08 p.c.) and the perfusate was collected both at room temperature and at 95° C. During 43 minutes before injection 85·5 c.c., and during 25 minutes after injection 39 c.c. of perfusate were collected. The unboiled portions were mixed with adenosine (1 in 100,000) and after 20 minutes incubation 55 p.c. of activity remained in the sample obtained after injection and in 40 minutes only 30 p.c. remained. The sample obtained before injection showed no loss of activity in 20 minutes, but there was a slight fall to 90 p.c. in 40 minutes probably to be explained by the presence of serum which was added to the sample obtained before injection in the same concentration as it could be present in the sample after injection were none to have been retained in the liver (1 c.c. in 39 c.c. perfusate). Drury, Lutwak-Mann and Solandt (1938) have shown that serum is capable of inactivating adenyl compounds. The perfusate collected at 95° C. contained adenyl compounds equivalent to 2·5γ adenosine per c.c. and 100 c.c. of the unboiled perfusate was capable of inactivating 0·5 mg. of adenosine in approximately 24 minutes. It is evident that enzyme is still liberated in considerable amount at quite low concentrations of chloroform where only traces of adenyl compounds can be detected.

It will be observed that with the injection of the large dose of chloroform the loss from the organ in adenylic acid is less than where smaller doses were used. This is due to the high concentration of chloroform since a mixture of such an order inhibits to some extent the inactivation of adenyl compounds. A piece of liver was ground up with silica and the extract divided into two equal parts. To one chloroform was added in the proportion of 0·1 c.c. to 5 gm. liver and to the other chloroform was added in a proportion of 0·004 c.c. to 5 gm. liver. The samples were left to stand at room temperature for one hour and then boiled. Another portion of the liver was extracted and immediately boiled. This extract contained adenyl compounds equivalent to 0·9 mg. adenosine per gm. Liver mixed with the large dose of chloroform contained 0·11 mg. adenosine per gm. and with the small dose 0·08 mg. adenosine per gm. In another experiment the content of the extract with the large dose of chloroform was equal to 0·65 mg. adenosine per gm. and with the small dose 0·4 mg. adenosine per gm.

(c) *Experiments on the intact dog.* Since it is possible that results obtained with the Tyrode-perfused organ might not pertain to the blood-filled organ, injections of chloroform were made into the portal vein in the intact dog heavily morphinized and under very light chloroform anaesthesia. Just before the injection was made a portion of liver was removed aseptically and weighed. The abdomen was closed and 24 hours later the entire liver was removed after quickly killing the dog by opening the thorax through the diaphragm from the abdomen. A portion of the liver was then weighed and extracted. A control experiment was performed where no chloroform was injected.

In one experiment a dog weighing 8 kg. was injected intraportally with 0·4 c.c. of chloroform and the liver weighed 247 gm. After the abdomen was closed the dog became very drowsy and eight hours later was semi-comatose. In twenty-four hours the condition of the dog was almost unchanged and on opening the abdomen the outer surface of the liver presented swollen dark and reddened areas irregular in outline. In between them the liver was pale. The cut surface revealed haemorrhagic areas and in between were fatty greasy yellowish patches. Portions of the haemorrhagic and the yellow areas were weighed and extracted together. These portions of liver contained adenyl compounds equivalent to 0·37 mg. adenosine per gm. The liver extract obtained before injection of chloroform contained adenyl compounds equivalent to 0·8 mg. adenosine per gm. The fall in content of adenyl compound is too large to be explained by oedema since the weight of liver (247 gm.) is within normal limits for a dog of this size.

The results of three other experiments are recorded in Table 2. It is seen that where no chloroform was injected (line 1) the content of the liver at the time of the first operation was 0·88 mg. adenosine per gm. while on the following day it was 0·79 mg. adenosine per gm. The difference in content is 10 p.c. which cannot be regarded as significant though the slight fall might have been due to the small amount of chloroform used in which case it is necessary to reduce the fall in content observed in the other experiments. As a further check one dog was operated upon under dial anaesthesia (4 c.c. used in all for an 8 kg. dog) and on the second day, as usual where chloroform was injected, no further anaesthesia was required. The fall in adenyl compound content in this case (* in Table 2) was comparable with the other cases. The fall where a con-

centration of 0·16 p.c. chloroform (c.c. chloroform to gm. liver injected) was attained was 54 p.c with 0·08 p.c. 49 p.c., and with 0·04 p.c. the fall was 46 p.c. It will be observed (Table 2) that there was a slight fall only in the histamine content of the organ which is almost within normal limits.

TABLE 2.

Liver content in adenyl compounds and histamine before and 24 hours after the injection intraportally of chloroform, in the intact animal.

Injection of Chloroform in c.c.	Weight of liver gm.	P.c. injection $CHCl_3$ v/w.	Liver Content. Adenyl compound mg. per gm. Before.	After.	Histamine γ per gm. Before.	After.
Nil	310	—	0·88	0·79	34	31
0·1	247	0·04	0·78	0·42	29	29
*0·2	250	0·08	0·45	0·23	20	16
0·4	245	0·16	0·80	0·37	50	44

* Dial anaesthesia.

Two dogs weighing 9·2 and 16·5 kg. were injected with 0·2 c.c. chloroform intraportally and soon became very drowsy. They both died overnight, so that a satisfactory figure for the adenyl compound content of the organ after injection was not obtained. It is possibly significant that the adenyl compound content of the liver in these animals before injection was very low, being equivalent to 0·29 mg. and 0·17 mg. adenosine per gm. respectively. On the other hand the histamine contents were relatively high, namely 45 and 30γ per gm. respectively. The concentration of injection of chloroform was approximately 0·08 p.c.

The Liberation of Histamine and S.R.S.

As well as titrating the outputs of adenylic acid on the guinea-pig heart preparation, tests were made upon the isolated jejunum of the guinea-pig and on the blood pressure of the atropinized cat. In this way outputs of histamine were estimated and confirmed. In every instance an output of histamine was detected, not however so high as the output of adenyl compound. There was also an output of a substance with an effect on the guinea-pig jejunum similar to that described by Feldberg and Kellaway (1938) and Feldberg, Holden and Kellaway (1938) as produced by the action of cobra venom on the isolated organ and upon egg-yolk.

TABLE 3.

Liberation of adenyl compounds and histamine from isolated perfused liver.

Dose $CHCl_3$ in c.c.	Weight of liver gm.	Content of liver. mg. Adenosine per gm.	γ Histamine per gm.	Output in perfusate. mg. Adenosine.	mg. Histamine.
Nil	49·8	0·25	57	Nil	Nil
0·07	73·9	0·25	54	0·08	0·03
0·12	111·3	0·7	83	0·36	0·08
0·15	52·4	0·7	110	0·22	0·11
1·0	49·9	0·4	57	3·4	0·38

In Table 3 are shown the results of four experiments and a control where the outputs of histamine and adenyl compounds are compared. The outputs in relation to time for the experiment of line 3 are shown in Fig. 3 and it is seen that the peak occurs at 3–10 minutes after injection for both. There was a secondary rise in the output of adenyl compound during second half-hour. The response of the isolated guinea-pig jejunum to unboiled samples (boiling markedly diminishes the response of the jejunum to liver S.R.S.) is shown in Fig. 4. In this experiment 0·2 c.c. of chloroform was injected into a dog's liver weighing 76 gm. and 32 c.c. of perfusate collected during 21 minutes before injection and 44 c.c. collected during 30 minutes after injection were obtained. At B is shown the response to 0·4 c.c. of the sample obtained after injection. The response is immediate owing to the presence of histamine, but a slow-reacting stimulant substance is shown to be present by the delay in relaxation after washing out. At A 0·4 c.c. of sample obtained before injection and at C 0·4 c.c. of Tyrode mixed with chloroform (0·2 c.c. chloroform to 44 c.c.) were without effect. The samples were left in contact with the gut for 30 seconds. The unlettered responses are to 0·1γ histamine.

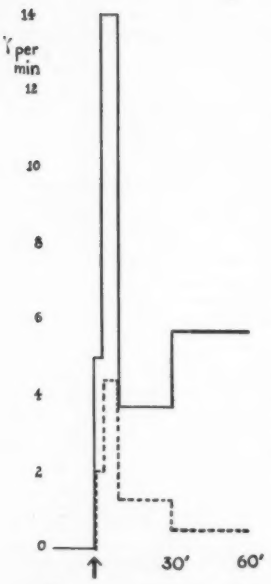


Fig. 3. Output of adenyl compound (continuous line) and histamine (interrupted line) from a perfused liver of a dog following the injection of 0·12 c.c. chloroform emulsified in 1 c.c. of dog serum (at arrow). Ordinates: output in γ per min.; abscissae: time in minutes. Details in text.

Experiments on Guinea-Pigs.

The liberation of acetylcholine. Gautrelet and Corteggiani (1938) have shown that acetylcholine is liberated from the acetylcholine complex in ground-up guinea-pig brain by cobra venom. This complex, however, is only associated with cellular material and cannot be distinguished from the remaining acetylcholine present in the cell, which is said to be in the "free" form, except by heat or acid extraction; such methods allow of more complete extraction by further disruption of cells not only of acetylcholine but also of histamine (Trethewie (1938)). Chloroform has been used to show the synthesis of acetylcholine in minced brain tissue (Stedman and Stedman (1937)), but these workers assumed that "mincing brain tissue causes a complete destruction of any acetylcholine which it may have contained," whereas actually incompletely ground-up tissue fragments retain their histamine and acetylcholine (Trethewie (1938)). It is not impossible that the effect of chloroform was simply one of liberation of acetylcholine from the cell and the contained acetylcholine was in the inactive form typical of pharmacologically active substances in general.

The injection of chloroform into the perfused head of the guinea-pig causes the liberation of a compound which produces contraction of the dorsal muscle of the leech and of the frog rectus. The outputs of acetylcholine so estimated in the perfusate are given in Table 4. It is also observed that the acetylcholine content of the guinea-pig brain after the injection of chloroform is less than the normal for these animals. In a series of experiments following the injection of chloroform (0·05–0·2 c.c.) the average content of the brain was shown to be 3·7γ acetylcholine per gm. (seven experiments), while for uninjected brains the average was 4·8γ acetylcholine (seven experiments). The standard deviation (S) in the former was 0·84 and in the latter 1·0. The mean difference was 1·1 and it can be computed that for the value S = 1·0 five pairs need to be compared, with S = 0·84 four pairs need to be compared to establish a 20 to 1 chance that this mean difference is significant (Snedecor (1938)). This requirement is satisfied since we have compared seven pairs. Furthermore, with larger doses of chloroform the mean difference was greater being 1·7γ for 0·2 c.c., 1·0γ for 0·1 c.c. and — 0·2γ acetylcholine per gm. for 0·05 c.c. chloroform injected. Therefore the lower content of injected brains is significant and is due in some way to the injection of chloroform. Furthermore, if one adds the content of the perfusate in acetylcholine to that of the brain the average content of the brain so estimated becomes 4·9γ per gm., which agrees closely with the experiments where chloroform was not injected the average content being 4·8γ per gm. The extreme variation in the form was 4·5–6·1γ and in the latter 4·8–6·5γ acetylcholine.

These findings are illustrated in the following experiment. A guinea-pig head weighing 136 gm. was perfused via both carotids with an eserinized Lock solution till the perfusate was clear. 23 c.c. of perfusate were collected in 11 minutes before the injection of 0·05 c.c. chloroform into each carotid. The perfusion flow slowed down considerably after injection and under increased pressure 25 c.c. of perfusate were collected during one hour. The perfusate was boiled and tested on the frog

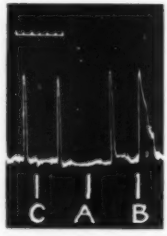


Fig. 4. Responses of the isolated jejunum of the guinea-pig to unboiled perfusate from a perfused dog's liver. The unlettered contractions are to 0·1γ histamine. Time in half-minutes. Details in text.

rectus. This is shown in Fig. 5. At A_{10} and A_{12} are shown the responses to acetylcholine, 1 in 10 and 1 in 12 millions. At 1 and C respectively no response was obtained to the sample collected before the injection or to the Locke solution to which chloroform was added (0·1 c.c. chloroform to 25 c.c.) and boiled. At 2 is shown the response to perfusate collected after the injection and it contained acetylcholine equivalent to 1: 11 millions.

TABLE 4.

The liberation of acetylcholine from the perfused head of the guinea-pig.

Injection $CHCl_3$ c.c.	Weight head gm.	Weight cerebrum gm.	Acetylcholine content cerebrum after injection (γ per gm.).	Output Acetylcholine in γ.
0·05	119	3·4	4·8	0·8
0·1	145	3·2	4·5	1·4
0·1	211	3·7	4·5	1·2
0·1	136	3·0	3·4	2·3
0·1	137	3·4	2·7	2·6
0·2	141	3·4	3·7	8
0·2	216	4·9	2·5	16
Nil	182	3·3	4·3	Nil

In addition histamine is liberated as is shown by contraction of the atropinized guinea-pig jejunum and also depression of the blood pressure in the atropinized cat with the vagi cut. The addition of histamine to the frog rectus and dorsal muscle of the leech preparation, in a concentration as is shown to be present in samples tested by the depression of the blood pressure of the atropinized cat and of the atropinized guinea-pig jejunum, is without effect.

Fig. 5. Responses of the isolated frog rectus to perfusate from the perfused head of a guinea-pig. Details in text.

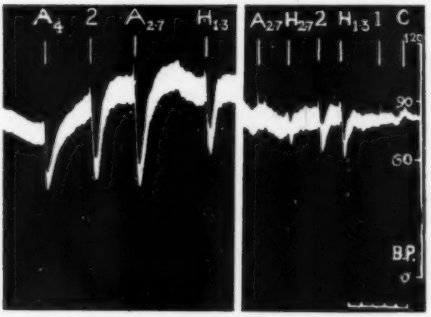


Fig. 6. Carotid blood pressure record from a cat weighing 2·5 kg. under ether and chloralose anaesthesia with the vagi cut. During the interval between the panels 1 mg. atropine was injected. Scale blood pressure in mm. Hg. Time in half-minutes. Details in text.

An endeavour was then made to titrate the acetylcholine and histamine content of samples of perfusate by testing on the guinea-pig jejunum and blood-pressure of the cat before and after the administration of atropine. In Fig 6 are shown the responses of the blood pressure of the cat to samples of perfusate from the experiment recorded above. At A_4, $A_{2·7}$ are shown the responses to acetylcholine 1: 4 and 1: 2·7 millions and at $H_{1·3}$, $H_{2·7}$ are shown the responses to histamine 1: 1·3 and 1: 2·7 millions. Between the panels 1 mg. atropine was injected. At *2* 2 c.c. of the sample obtained after injection of chloroform (boiled) and at *1* 2 c.c. of the sample before injection (boiled) were tested. It is seen that before atropine administration sample *2* is intermediate in content between 1: 4 and 1: 2·7 millions acetylcholine (= 1: 3·2 millions) and that histamine 1: 1·3 millions is equivalent to acetylcholine 1: 4 millions. This gives the histamine value for the depression of *2* as 1: 1 million. After atropine the value is reduced to 1: 1·5 million.

Interpolating: *2* = 1: 3·2 millions acetylcholine.
= 1: 1 million histamine.

after atropine *2* = 1: 1·5 million histamine.

Thus one-third of the concentration before atropine was not due to histamine and was evidently due to acetylcholine, and therefore the concentration of acetylcholine in this samples is 1: 10 millions (actually 1: 9·6). This agrees very closely with the figure found for the leech preparation, 1: 11 millions. At C in Fig. 6 2 c.c. of Tyrode solution to which chloroform was added (0·1 c.c. chloroform to 25 c.c.) and boiled was without effect.

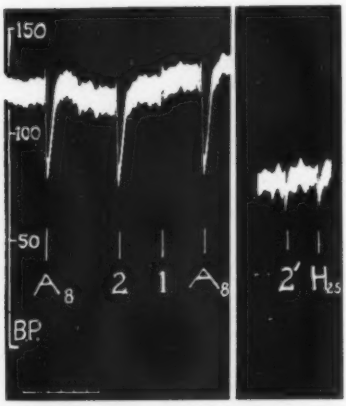


Fig. 7. Carotid blood pressure record from a cat weighing 2 kg. under ether and chloralose anaesthesia with the vagi cut. During the interval between the panels 1 mg. atropine was injected. Scale blood pressure in mm. Hg. Time in half-minutes. Details in text.

In another experiment where a guinea-pig 's head weighing 145 gm. was perfused through both carotids, 17·5 c.c. of perfusate were collected for 15 minutes before the injection of 0·05 c.c. chloroform into each carotid and 14 c.c. were collected after the injection for 1 hour. The perfusate after injection when boiled and tested on the cat 's blood pressure caused a depression equivalent to 1: 4 millions acetylcholine. The sample of perfusate collected before injection was without effect. After atropine the response to acetylcholine was abolished but the sample obtained after injection still caused depression of blood pressure equivalent to 1: 2·5 million histamine ($H_{2·5}$ in Fig. 7). Testing on the atropinized guinea-pig jejunum the sample contained histamine equivalent to 1: 2 millions. In Fig. 7 are shown the responses at A_8 to 2 c.c. acetylcholine 1: 8 millions, at 2 to 2 c.c. of the sample of perfusate obtained after the injection of chloroform diluted 1 in 2 and at 1 to 2 c.c. of the sample obtained before injection similarly diluted. In the interval between the panels 1 mg. atropine was given. At 2' 2 c.c. of the sample obtained after injection was tested undiluted. In Fig. 8 are shown the responses of the frog rectus preparation to these samples. At A_{40} and A_{50} 1 in 40 and 1 in 50 millions acetylcholine respectively were tested. The response at 2 to the sample obtained after injection boiled and then diluted 1 in 4 with diluted Locke solution was equivalent to 1 in 40 millions acetylcholine. At 1 the sample obtained before injection boiled and diluted 1 in 4; at H histamine 1 in 2 millions and at C chloroform in diluted Locke (0·1 c.c. chloroform to 19·5 c.c.) and boiled were all without effect.

The outputs of acetylcholine as measured by the leech preparation and the frog rectus can be accepted as probably correct, but since gut- and blood-pressure determinations are complicated by the presence of histamine, and further since the contraction on one hand and the depression on the other do not progress arithmetically for arithmetic increases of dosage, the attempt to titrate them by the use of atropine is not entirely sound. Nevertheless the presence of more contraction on the one hand and more depression on the other produced by samples after injections of chloroform in proportion to a known concentration of histamine, and their reduction to less than that known concentration of histamine after the administration of atropine definitely reveals the presence of a substance producing contraction of the guinea-pig jejunum and depression of the cat's blood-pressure which is inhibited by atropine.

Fig. 8. Responses of the isolated frog rectus to perfusate from the perfused head of a guinea-pig. Details in text.

In order to overcome the difficulty of the presence of histamine in the perfusate when testing on the cat 's blood pressure a head was perfused at a more rapid rate following injection so as to dilute the liberated histamine. 0·1 c.c. of chloroform was injected and 41 c.c. of perfusate were collected over sixty minutes. This perfusate was boiled and was without effect on the cat 's blood pressure following the injection of atropine, even in a dose of 5 c.c. On the non-atropinized cat a

depression of blood pressure equivalent to acetylcholine 1 in 18 millions was obtained. On testing this sample on the frog rectus the activity in acetylcholine was 1 in 16 millions. This sample also produced inhibition of the auricle in the guinea-pig heart preparation which was abolished by atropine.

It is possible that some of the acetylcholine estimated in the perfusate was liberated not from nervous tissue but from muscle, though the latter contains acetylcholine in very meagre amounts, being equivalent to 0·1–0·25γ acetylcholine per gm.

DISCUSSION.

It has been shown that for surgical anaethesia the concentration of chloroform required in the tissues is 0·04 p.c. (=0·03 p.c. vol. liquid to weight tissue) but that in practice higher concentrations are employed (Clark (1938)). The studies here recorded demonstrate injury to the liver when concentrations of 0·04–1·9 p.c. chloroform were used. It therefore seems evident, if one accepts the findings obtained in the isolated organ perfused with Tyrode as significant, that in the range of concentrations used in anaesthesia a biochemical change can be detected, as shown by the liberation of adenyl compounds and of an inactivating enzyme. Further, in delay experiments on the intact dog, a considerable loss of adenyl compounds could be detected in the liver after an interval of 24 hours, with concentrations of chloroform ranging from 0·04 to 0·16 p.c.

In two dogs where chloroform was injected into the portal vein to give a concentration of 0·08 p.c., death occurred in 10–24 hours following on increasing drowsiness in the one and a period of consciousness in the other. The livers of these dogs were both very low in adenyl compound (equivalent to 0·17 and 0·29 mg. adenosine per gm.) which is possibly significant since where the same dose and twice this dose were injected into two other dogs, though drowsiness was produced both were alive in 24 hours and their livers contained adenyl compound equivalent to 0·45 and 0·8 mg. adenosine per gm. If one compares the ratio of chloroform injected (c.c.) in the intact dog to the adenyl compound content of the liver as mg. adenosine per gm. the figures obtained are nil, 0·1, 0·4, 0·5, 0·7, and 1·2. Where the ratio was 0·7 and 1·2 the dogs died. It may be worthy of note that the content of the dog's liver in adenyl compound is more variable than has been found for the rabbit. The variation here was 0·17 to 0·9 mg. adenosine per gm. Dogs are obtained from widely different sources and they have not been maintained under the same conditions.

We believe the substance liberated from the perfused head which causes contraction of the dorsal muscle of the leech and of the frog rectus is acetylcholine. The evidence for this lies not only in the fact that the concentration of the brain in acetylcholine is less following the injection of chloroform, and that Stedman and Stedman (1937) have shown that the substance in the brain which causes contraction of the leech muscle is acetylcholine, but also from pharmacological assay of the outflowing perfusate. It was shown that a substance was liberated which causes contraction of the frog rectus and the dorsal muscle of the leech, and that these preparations were insensitive to histamine and adenyl compound in the concentration in which they might possibly have been present in the perfusate. It was also shown that the substance liberated caused contraction of the guinea-pig jejunum and depression of the blood pressure of the cat which were inhibited by atropine. Furthermore, in an experiment where the concentration of histamine liberated was so lowered by a more rapid perfusion rate that it caused no depression of the blood pressure of the atropinized cat even in a dose of 5 c.c., there was agreement within 11 p.c. between the concentration of acetylcholine estimated on the blood-pressure of the cat and on the frog rectus, and also this sample produced inhibition of the right auricle of the guinea-pig heart preparation which was abolished by atropine.

SUMMARY.

Chloroform causes the liberation of adenyl compounds and an inactivating enzyme, histamine and S.R.S. from the isolated perfused dog's liver.

There is a loss of adenyl compound from the liver of the intact dog following the injection of chloroform intraportally.

Acetylcholine is liberated by chloroform from the isolated perfused head of the guinea-pig.

REFERENCES.

Bennett, D. W. and Drury, A. N. (1931): J. Physiol., 72, p. 288.
Chang, H. C. and Gaddum, J. H. (1933): *Ibid.*, 79, p. 255.
Clark, A. J. (1938): Applied Pharmacology, 6th Ed. [Churchill], p. 201.
Drury, A. N., Lutwak-Mann, C. and Solandt, O. M. (1938): Quart. J. exp. Physiol., 27, p. 215.
Feldberg, W., Holden, H. F. and Kellaway, C. H. (1938): J. Physiol., 94, p. 232.
Feldberg, W. and Kellaway, C. H. (1937): Austral. J. exp. Biol., 15, p. 461.
Feldberg, W. and Kellaway, C. H. (1938): J. Physiol., 94, p. 187.
Feldberg, W. and Krayer, O. (1933): Arch. exp. Path. Pharmak., 172, p. 170.
Gautrelet, J. and Corteggiani, E. (1938): C. Rend. des Séances de L'Acad. des Sciences, 217, p. 465.
Kellaway, C. H. and Trethewie, E. R. (1939): Austral. J. exp. Biol., 17, p. 225.
Kellaway, C. H. and Trethewie, E. R. (1940): *Ibid.*, 18, p. 63.
Minz, B. (1932): Arch. exp. Path. Pharmak., 168, p. 292.
Snedecor, G. W. (1938): Statistical Methods, Iowa, p. 70.
Stedman, E. and Stedman, E. (1937): Biochem. J., 31, p. 817.
Trethewie, E. R. (1938): Austral. J. exp. Biol., 16, p. 225.

THE BACTERIOLOGY, GROWTH FACTOR REQUIREMENTS AND FERMENTATION REACTIONS OF *CLOSTRIDIUM ACETOBUTYLICUM* (WEIZMANN)

by SYDNEY D. RUBBO, MARGARET MAXWELL, R. A. FAIRBRIDGE, AND J. M. GILLESPIE

(Department of Bacteriology, University of Melbourne).

(Accepted for publication 13th July, 1941.)

The butyl alcohol-acetone fermentation involves the conversion of simple or complex carbohydrates into solvents and gaseous products by *Clostridium acetobutylicum* (Weizmann), and nearly related anaerobes. The systematic studies of McCoy, Fred, Peterson and Hastings (1926), Weyer and Rettger (1927), and the serological work of McCoy and McClung (1935) established *Cl. acetobutylicum* (W.) as a species of the genus *Clostridium*. The findings of these authors clarified the chaotic state of previous systems of nomenclature of the butyl organisms and formed the basis of the description of *Cl. acetobutylicum* (W.) given by Bergey (1939). However, it is still difficult to isolate and identify the organism by purely bacteriological methods.

The object of this investigation was to obtain further data relating to *Cl. acetobutylicum* (W.) which could be used for its isolation and identification. These findings are described in Part I. In Part II the isolation of a factor essential for growth of *Cl. acetobutylicum* (W.) in synthetic medium is described. This factor was identified as *p*-aminobenzoic acid by Rubbo and Gillespie (1940), and although it is adequate for growth the presence of a second factor, *acetone factor*, is required for the production of acetone. The *acetone factor*, described in Part III, is a heat stable substance precipitated by phosphotungstic acid from a yeast extract and alone is incapable of supporting growth in the synthetic medium. Its chemical identity is unknown.

Finally, in Part IV, a normal fermentation curve for wheat mash is shown in which the yields of solvents and titratable acidity are followed during fermentation. Attempts to increase the yields or rate of formation of solvents with the view to increasing the efficiency of the industrial fermentation are also detailed.

Origin of cultures. Twelve organisms were used in this study. Seven were isolated here and identified as *Cl. acetobutylicum* (W.); two strains were obtained from the American Type Culture Collection (Nos. 862 and 4259), whilst the remaining three, although butyl alcohol-acetone fermenters, differed from *Cl. acetobutylicum* (W.) in that they were chromogenic. Two cultures of *Granulobacter pectinovorum* (A.T.C.C. Nos. 6014 and 6015) were originally included, but as they failed to produce solvents in wheat mash fermentation they were discarded.

In the isolation of the Australian cultures a wide range of raw materials was examined including soil (86 samples), potatoes (35 samples), various cereals and vegetables. Potatoes were found to be the most reliable source of the organism, whilst soil was quite unsuitable because of the paucity of *Cl. acetobutylicum* (W.) contained therein and the overwhelming number of unrelated spore formers present. Five of the cultures of *Cl. acetobutylicum* (W.) isolated were recovered from potatoes, one from wood pulp, and one from maize.

PART I. ISOLATION AND SYSTEMATIC BACTERIOLOGY OF *CL. ACETOBUTYLICUM* (W.).

Method of isolation. Tubes of 6 p.c. wheat mash were heated in boiling water bath for twenty minutes to expel dissolved air and then inoculated with small portions of the raw material. The tubes were allowed to stand in the air for 1 minute, cooled in water and incubated at 37° C. Tubes showing a typical fermentation in 48 hours were subcultured after 7 days incubation into wheat mash, using an inoculum of about 1 ml. After incubation, again for 7 days, three drops of culture were spread over *wheat-extract agar* plates, which were then dried and incubated at 37° C., aerobically and anaerobically. After 2 or 3 days incubation typical discrete colonies

were picked off the anaerobic plate and inoculated into wheat mash. The process of plating was repeated until no growth was obtained on the aerobic plates. Usually three anaerobic platings were sufficient to separate the organisms from aerobic sporing bacilli. The pure cultures were maintained in wheat mash and replated periodically to ensure purity.

Wheat-extract agar medium. In the early stages of this work, difficulty was experienced in growing the organism on the surface of solid media. Despite repeated attempts, no growth was obtained on blood agar, or on meat infusion agar, and with some degree of uncertainty on glucose agar and the malt extract agar of Weyer and Rettger (1927). After some experimentation a *wheat-extract agar* medium was developed. This was prepared as follows: 50 gm. of wheat meal are heated in the autoclave with 500 ml. of N/10 HCl at 10 lb. pressure for 30 minutes. The mixture is filtered, neutralized with 2 N.NaOH and the pH adjusted to 7·0. Water is added to give 1,000 ml., 20 gm. of agar are added, dissolved by heat, filtered, tubed and sterilized in the autoclave. This medium is practically clear and gives good growth and colony formation of *Cl. acetobutylicum* (W.). Furthermore, it is selective and most other anaerobes and sporing aerobes fail to grow on it under anaerobic conditions.

Morphology.

Cl. acetobutylicum (W.) is a motile, Gram positive, rod-shaped organism with rounded ends, straight or slight curved axis and parallel sides. Stained preparations of young wheat-mash cultures show a characteristic palisade arrangement (Fig. 1), modal size 5μ by ·5μ. Sporing forms are typically clostridial in shape (Fig. 2).

Young cells stain yellow with iodine whilst clostridial forms stain blue or with blue granules.

Fig. 1. Organisms stained by Gram from 24 hour wheat mash culture showing palisade arrangement. (Magnification × 1,000.)

Fig. 2. Organisms stained by Gram from 48 hour wheat mash culture showing clostridial forms but no palisade arrangement. (Magnification × 1,000.)

Cultural Characteristics.

The best medium for cultivation is wheat or maize mash in which the organisms grow profusely even under aerobic conditions. Unless otherwise stated all other cultures were incubated in a McIntosh and Fildes' anaerobic jar.

6 p.c. Wheat mash medium: 2 days at 37° C. The gel is liquefied in 24 hours during which copious amounts of gas are evolved raising undigested portions of the wheat above a turbid fluid (Fig. 3B). Thick "streamers" of organisms are frequently seen hanging from this head. As fermentation ceases, the head falls leaving a clear, sweet-smelling supernatant fluid (Fig. 3C).

Wheat extract agar: 2 days at 37° C. Colonies are irregular to circular in shape, convex to umbonate, about 2 mm. in diameter, entire margin, creamy-white, opaque, smooth glistening surface (Fig. 4). The consistency is viscid. If the plate is not dried before incubation, the characteristic spreading growth of anaerobes is obtained.

Nutrient agar plate: 4 days at 37° C. No growth.

2 p.c. Glucose agar plate: 2 days at 37° C. Growth inconstant, but when present similar to that on wheat-extract agar.

Blood agar plate: 4 days at 37° C. No growth. Wheat extract agar containing 10 p.c. blood allows good growth. No haemolysis, but blood is discoloured.

Nutrient broth: 4 days at 37° C. No growth.

2 p.c. Glucose broth: 2 days at 37° C. Prolific growth aerobically if tubes are boiled prior to inoculation. Uniform turbidity, thick sediment, and much gas is evolved.

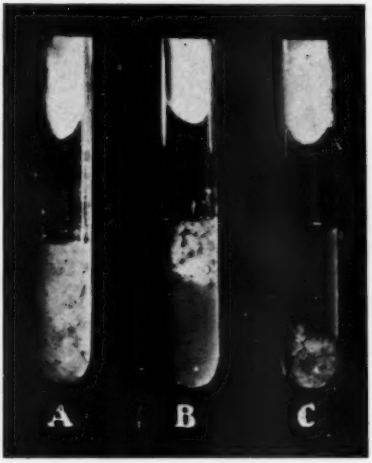


Fig. 3. Wheat mash cultures: A. uninoculated, B. after 24 hour incubation showing head of insoluble fibre and gas formation, C. same culture after 48 hours incubation showing deposit of insoluble fibre and clear supernatant fluid.

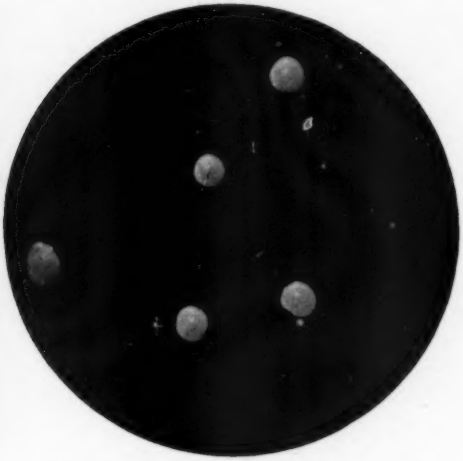


Fig. 4. Colonies on *wheat-extract* agar after 72 hours incubation at 37° C. anaerobically. The colonies are circular, opaque with a pearly-like lustre. (Magnification × 3.)

Cooked meat medium: 4 days at 37° C. aerobically. Growth saccharolytic in type, the gas evolved raising meat to a head. No foul odour detectable.

Gelatin: 4 days at 37° C. and subsequently cooled in ice. Liquefaction.

Potato slopes: 2 days at 37° C. Cream coloured growth, digestion, gas and solvent odour.

Biochemical Reactions.

In a basal medium containing yeast extract, Stage 1 (see later), acid and gas are formed in glucose, galactose, mannose, fructose, xylose, arabinose, sucrose, lactose, maltose, mannitol, starch, salicin, inulin, dextrin, glycogen.

Glycerol, ducitol, sorbitol, adonitol, inosite, rhamnose, trehalose and raffinose are not fermented. Some strains slightly ferment the last three sugars.

V.P. test: Positive with several sugars. *Indol:* Negative. *Nitrate broth:* Not reduced to nitrite. *Litmus milk:* Milk is clotted with disruption—a typical stormy clot—litmus is reduced.

Antigenic Structure.

Antisera prepared against "O" suspension of one Australian and one American strain (A.T.C.C. No. 862) agglutinated all strains used in this study without significant difference of titre.

Pigmented Butyl Alcohol-Acetone Fermenters.

Three strains isolated in this study were distinguishable from *Cl. acetobutylicum* (W.) by the production of a pigmented growth. Cultures of strain 9 were coloured a bright orange whilst strains 8 and 10 were flesh pink. In addition to pigment production these strains differed in failing to ferment mannitol, and were agglutinated only to a low titre by antisera prepared against *Cl. acetobutylicum* (W.). On the other hand, they produced normal yields of butyl alcohol and acetone in wheat mash fermentation.

Discussion.

The above description of *Cl. acetobutylicum* (W.) was based on a study of the modal behaviour of nine strains isolated from widely different sources. Cultural and other characters distinguish this bacterium from other members of the genus without recourse to chemical analyses of the products of fermentation. It is not suggested, however, that chemical methods should be discarded, but that they should only be applied to those cultures which possess certain distinctive features.

Thus, the palisade arrangement of the organism in 24 hour wheat mash cultures and the clostridial forms, lacking in this arrangement, but seen on further incubation, are important features of morphology. The growth in wheat mash is always vigorous during which copious quantities of gas are liberated. Certain features of this growth are typical of the organism, namely, the rise of a head of undigested wheat in 24 hours frequently with attached pendant streamers of organisms and, on further incubation, the sinking of this head. Pigments, orange and pink, should be absent and a sweet solvent odour is detectable in older cultures. The growth on *wheat extract agar* is typical in that this medium is selective for the bacterium which develops on it as circular creamy-white viscid colonies. Furthermore, the inability of the organism to grow in nutrient broth or on blood agar plates anaerobically distinguishes it from many members of the genus *Clostridium*. Bergey (1939), following McCoy *et alii* (1926), describes growth on blood agar but we have been unable to confirm this statement. In conclusion, agglutination with "O" antisera separates *Cl. acetobutylicum* (W) from other clostridia, including the pigmented butyl alcohol-acetone organisms.

PART II. GROWTH FACTOR REQUIREMENTS OF *CL. ACETOBUTYLICUM* (W.).

Brown *et alii* (1938) showed that an acidic ether-soluble extract of Difco yeast extract was essential for vigorous growth of *Cl. acetobutylicum* (W.) in a medium of known chemical constitution. Further work by these authors (1939) showed that known growth factors—thiamin, riboflavin, cystine, nicotinic acid, pimelic acid, pantothenic acid, β alanine and uracil did not stimulate growth. Weizmann and Rosenfeld (1937) concluded that at least one growth factor present in yeast dialysate was required. These authors (1939) stated that one of the active growth factors required for *Cl. acetobutylicum* (W.) is biotin which brings the nutritive level of a synthetic medium up to 70 p.c. of the nutritional level represented by a maize mash. More recently, Oxford, Lampen and Peterson (1940) concluded that two growth factors were required for *Cl. acetobutylicum* (W.), viz. biotin and another factor. This second factor, BY factor, was recovered from yeast and had the properties of a weak acid whose activity was destroyed by nitrous acid. Rubbo and Gillespie (1940) isolated *p*-aminobenzoic acid as the benzoyl derivative from brewer's yeast and found that both the naturally occurring acid, and a synthetic sample, were able to stimulate the growth of the bacterium in a medium of known chemical constitution. We have now extended our previous observations to a wider range of chemical types some of which are closely related to *p*-aminobenzoic acid and others which are entirely unrelated. The findings confirm our previous conclusion, namely, that *p*-aminobenzoic acid and structurally related compounds (see Tables 2, 3) function as growth factors for *Cl. acetobutylicum* (W.). We can only reconcile our findings with those of other authors by tentatively assuming that Weizmann's biotin preparation and the BY factor were crude extracts containing small amounts of *p*-aminobenzoic acid.

Experimental.

A preliminary survey of many diverse types of raw materials as sources of growth factor revealed that its distribution was widespread. Extracts of wheat, potatoes, tomatoes, marmite, Lab. Lemco, and yeast were all found to possess growth factor activity. Brewer's yeast was selected for large scale work on account of its cheapness and the ease with which it could be handled. At each stage in the isolation from yeast the activity of the product was assessed

ERRATUM.

Page 189. In lines 8, 9 the concentrations of inorganic salts in the basal medium should read one-tenth of those stated in each case.

fairly quantitatively in the manner detailed below. It will be seen that 30 kg. of wet brewer's yeast yielded 10 mg. of benzoyl derivative of the growth factor, which proved to be *p*-aminobenzoic acid. This yield is roughly equivalent to 1 part of *p*-aminobenzoic acid in 6 million parts of wet yeast.

The technique used for determining the growth factor content of all extracts examined was as follows. A basal medium of known chemical constitution containing glucose as a source of carbon and asparagine as sole source of nitrogen was selected. This medium had the following composition: asparagine 0·1 p.c., glucose 2·0 p.c., KH_2PO_4 0·5 p.c., K_2HPO_4 0·5 p.c., $MgSO_4$ 0·2 p.c., NaCl 0·1 p.c., $FeSO_4.7H_2O$ 0·01 p.c., $MnSO_4.4H_2O$ 0·01 p.c. in distilled water. A solution of glucose was sterilized separately to avoid decomposition whilst the other constituents were dissolved in distilled water and the solution adjusted to pH 6·8 before autoclaving. The sterile solutions were mixed aseptically before use and the basal medium was then tubed in 5 ml. quantities. Tenfold dilutions of aqueous solutions of crude extract covering a range from 10·0 mg./ml. to 0·10μ gm./ml., and with purified extracts from 0·10μ gm./ml. to 0·00001μ gm./ml. were added in 0·5 ml. amounts to tubes of basal medium. Dilutions of each extract were tested in duplicate by inoculation with 0·1 ml. of a 24 hour wheat mash culture. Control tubes of basal medium without added extract (to determine the carry-over of any growth factor with the inoculum), and of 6 p.c. wheat mash (to test the viability of the inoculated bacteria) were included in every experiment. The test media were incubated in an anaerobic jar at 37° C. for 48 hours and the results read. Growth factor was assumed to be present in those tubes in which active growth, as evidenced by turbidity and gas production, was observed. In the later stages of purification a positive result was only recorded when growth was sustained in at least four serial subcultures in the same medium. The purity of this growth was frequently checked by staining. In no instance has the basal medium alone given growth of the organisms.

Isolation of Para-aminobenzoic Acid from Yeast.

To facilitate the recording of these results quantitatively the term—"unit of growth factor activity" (= G.F.A. in future)—has been introduced, *one unit being the smallest amount of material necessary to stimulate visible growth of Cl. acetobutylicum* (W.) *in 5 ml. of basal medium under the conditions stated above.* It has been consistently noted in this work that 100 units are required to give optimal growth of the organism.

Stage I. One kg. of wet brewer's yeast, which had been washed twice with water, was suspended in 2,000 ml. of 0·3 N.H_2SO_4 and autoclaved at 10 lb. pressure for 40 minutes. The mixture was allowed to stand, then strained through butter muslin and neutralized with 10 N.NaOH. A precipitate formed and was filtered off. The filtrate (1,500 ml.) was evaporated to 200 ml.

G.F.A.: Precipitate .. .. inactive.
Filtrate .. .. 1 unit in 25 μ gm.

Stage II. Four volumes (800 ml.) of alcohol were added to the concentrated filtrate with constant stirring. After standing some hours the precipitate was filtered off and washed with 80 p.c. alcohol. The washings were added to the filtrate and the whole evaporated to dryness and redissolved in 200 ml. of water.

G.F.A.: Precipitate .. .. inactive.
Filtrate .. .. 1 unit in 4 μ gm.

Stage III. The filtrate was brought to pH 6·0 and extracted by ten 2-minute hand shakings with wet butyl alcohol. The aqueous phase was separated and the solvent dehydrated *in vacuo* to 200 ml. (Experiment showed that pH 6·0 was optimal for the above extraction.)

G.F.A.: Aqueous phase .. .. inactive.
Butyl alcohol phase .. .. 1 unit in 1·2 μ gm.

Stage IV. To the anhydrous butyl alcohol solution an equal volume of ether was added. A large amount of brown gum was precipitated and removed. Solvents were distilled off *in vacuo* and the residue dissolved in 100 ml. of distilled water.

G.F.A.: Ether precipitate . .. . slight activity.
Solvent phase .. .. 1 unit in 0·3 μ gm.

Stage V. The aqueous solution was treated with 10 p.c. phosphotungstic acid dissolved in 6 p.c. H_2SO_4 till no further precipitate developed. The precipitate was removed by centrifuging and the supernatant treated with baryta. Excess baryta was precipitated with N.H_2SO_4. The solution was evaporated to dryness and extracted with hot absolute alcohol for 24 hours after which the alcoholic solution was evaporated to dryness and dissolved in 40 ml. of water.

G.F.A.: V.B. = Phosphotungstic precipitate .. .. inactive.
Alcohol insoluble fraction of supernatant .. .. inactive.
V.A. = Alcohol soluble fraction of supernatant 1 unit in 0·06 μ gm.

Stage VI. The aqueous solution at pH 7·0 was precipitated with saturated basic lead acetate. The precipitate, which carried down most of the colouring matter of the solution, was separated in a centrifuge and washed with 1 p.c. basic lead acetate. The supernatant and washings were acidified with H_2SO_4 and de-leaded with H_2S. The filtrate, after boiling to remove H_2S, was adjusted to pH 7·0 and concentrated to 40 ml.

G.F.A.: Lead precipitate .. .. inactive.
Supernatant .. .. 1 unit in 0·01 μ gm.

Stage VII. At this stage the concentrates from 30 separate batches were combined thus providing 1,200 ml. of a partially purified and decolourized solution of the growth factor extracted from 30 kg. of brewer's yeast.

To the mixed batches from stage VI 10 p.c. $HgSO_4$ in 1 p.c. H_2SO_4 was added till no further precipitate developed. This precipitate was buff coloured and was removed by centrifuging. The precipitate was washed several times with 1 p.c. $HgSO_4$. It was resuspended in water, decomposed with H_2S. After filtration the solution was evaporated to dryness.

G.F.A.: Mercury precipitate .. .. 1 unit in 0·01 μ gm.
Mercury supernatant .. .. inactive.

Stage VIII. The powder from stage VII was extracted with hot alcohol for 24 hours and the alcoholic extract evaporated to dryness. The alcohol soluble residue was dissolved in 10 ml. of water acidified to pH 3·5 and then extracted with ether in a continuous extractor for 48 hours. The ether fraction was concentrated to a clear yellow oil weighing 105 mg.

Experiment showed the presence of a primary aromatic amine in the oil. Since sodium nitrite destroyed the growth factor activity of this extract an aromatic amine was suspected as being the active constituent.

G.F.A.: Alcohol insoluble residue .. .. inactive.
Alcohol and ether soluble oil .. .. .. 1 unit in 0·001 μ gm.

Stage IX. Five ml. of an aqueous solution containing 100 mg. of oil was benzoylated in the usual manner with benzoyl chloride and sodium hydroxide. An alcoholic solution of a crude benzoyl derivative (10 mg.) was decolourized with a small quantity of Norit and recrystallized five times from alcohol. The white crystallized product was identified as *p*-benzoylaminobenzoic acid by the following tests:

Melting point of benzoyl derivative	277° C.
Melting point of *p*-benzoylaminobenzoic acid	278° C.
Mixed melting point	277° C.
Molecular weight of benzoyl derivative (by cryoscopic method)	238–239
Molecular weight of *p*-benzoylaminobenzoic acid	241

Yield of *p*-benzoylaminobenzoic acid
= 10 mg. of crude substance from 30 kg. wet yeast.
= 5 mg. of *p*-aminobenzoic acid.

Some idea of the fate of the growth factor can be gathered by analysis of Table 1 in which an assessment of the total number of units per 1 kg. of wet yeast has been made at each stage in the isolation. It will be seen that as the weight of crude extract recoverable from 1 kg. of yeast diminishes, the growth factor activity increases proportionately. The figures, which are rough approximations show a 25,000-fold concentration of the factor from stages I to IX and a 6 million-fold concentration from wet yeast.

TABLE 1.

Isolation of p-aminobenzoic acid from brewer's yeast.

Yeast extract.	Yield from 1 kg. gm.	Growth factor activity 1 unit in — μ gm.	Total units per 1 kg.
Stage I	75·0	25·0	$3·0 \times 10^6$
„ II	11·2	4·0	$2·8 \times 10^6$
„ III	3·0	1·2	$2·5 \times 10^6$
„ IV	0·6	0·3	$2·0 \times 10^6$
„ V	0·13	0·06	$2·1 \times 10^6$
„ VI	0·03	0·01	$3·0 \times 10^6$
„ VII	0·06	0·01	$6·2 \times 10^6$
„ VIII	0·003	0·001	$3·3 \times 10^6$
„ IX *p*-benzoyl-amino-benzoic acid	0·0003	0·001	$0·3 \times 10^6$

Control.

Synthetic *p*-benzoylaminobenzoic acid	0·001
Synthetic *p*-aminobenzoic acid	0·0001

The loss of the factor at stage IX in which the total number of units per 1 kg. of wet yeast is reduced to $0{\cdot}3 \times 10^6$ is an apparent but not a real loss. This is due to the fact that the estimation was made on the benzoyl derivative which, as also shown in the Table, has only one-tenth the activity of *p*-aminobenzoic acid.

Growth Factor Activity of Substances Structurally Related to Para-aminobenzoic Acid.

The discovery of the growth stimulating activity of *p*-aminobenzoic acid facilitated an extended investigation in which the connection between molecular pattern and growth stimulation could be followed. To this end a series of 35 organic chemicals were examined some of which were closely related to *p*-aminobenzoic acid whilst the other compounds were entirely dissimilar. It is now evident that substances conforming to the structural configuration of *p*-aminobenzoic acid are active. These findings are of particular interest in view of the anti-sulphanilamide properties assigned to a similar series of compounds by Woods (1940). The compounds which Woods found to be anti-sulphanilamide in action, (e.g. *p*-aminobenzoic acid) we have shown to be active as growth factors, whilst those incapable of supporting growth (e.g. *p*-hydroxybenzoic acid) are inactive as anti-sulphanilamide agents. Our confirmation of Woods' experiments on the mode of action of sulphanilamide has been described separately by Rubbo and Gillespie (1941).

Since *Cl. acetobutylicum* (W.) is a sporing bacterium it was possible to demonstrate that spores responded, or failed to respond, to the same accessory factors as did the vegetative forms.

Experimental. Seven ten-fold dilutions in water of each test substance in concentrations varying from $20{\cdot}0\ \mu$ gm./ml. to $0{\cdot}00002\ \mu$ gm./ml. were added in 0·5 ml. amounts to 5 ml. of basal medium (page 189). The aqueous dilutions were sterilized either by autoclaving or by filtration. The suitability of each so-constituted medium was determined by inoculation with 0·1 ml. of a 24 hour wheat mash culture of *Cl. acetobutylicum* (W.). Control tubes were included as before and growth was noted after 48 hours incubation at 37° C. in an anaerobic jar.

TABLE 2.

Growth factors for Cl. acetobutylicum *(W.).*

No.	Substance.	Solution sterilized by	Growth factor activity (G.F.A.) 1 unit in
1.	*p*-aminophenyl acetic acid	A. and F.	$0{\cdot}00001\ \mu$ gm.
2.	*p*-aminobenzoic acid	A. and F.	0·0001 „
3.	*p*-aminobenzoic acod (Na^+ salt)	A.	0·0001 „
4.	*p*-aminobenzoic acid (ethyl ester)	A.	0·0001 „
5.	*p*-aminobenzaldehyde	A. and F.	0·0001 „
6.	*p*-aminobenzoic acid (benzoyl derivative)	F.	0·001 „
7.	*p*-aminobenzoic acid (diethylaminoethyl ester)	A. and F.	0·001 „
8.	*p*-nitrobenzoic acid	A.	0·001 „
9.	*p*-nitrobenzaldehyde	F.	0·01 „
10.	*p*-methylbenzoic acid	A.	1·0 „
11.	*o*-aminobenzoic acid	A.	10·0 „
12.	*m*-aminobenzoic acid	A.	10·0 „
13.	*p*-aminobenzamide	A.	10·0 „

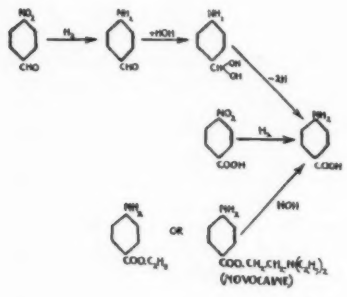
Inactive.

14. *p*-hydroxybenzoic acid, 15. *p*-aminobenzyl alcohol*, 16. *p*-aminophenol*, 17. *p*-dimethylaminobenzaldehyde, 18. *p*-aminobenzenesulphonamide, 19. aniline, 20. benzamide, 21. benzene, 22. benzoic acid, 23. nicotinic acid, 24. ascorbic acid, 25. pimelic acid, 26. glutamic acid, 27. tryptophane, 28. cysteine, 29. tyrosine, 30. leucine, 31. glycine, 32, cholesterol, 33. vitamin A, 34. thiamin, 35. inositol.

* Solution sterilized by F, others by A.

F. Solution filtered through Seitz E.K. asbestos pad.
A. Solution autoclaved at 10 lb. pressure for 15 minutes.

It will be seen, *vide* Table 2 above, that those substances failing to support growth in a concentration of 10 μ gm./5 ml. medium, the highest concentration used, are considered to be inactive. This figure is admittedly small representing approximately 10^{-5} M concentration, and it is possible that stronger concentrations of some compounds may support growth. In these experiments, however, it was not practicable to modify our technique to cover a greater range of concentrations. In any case, differences of great magnitude have been demonstrated, for instance *p*-aminobenzoic acid is 10,000 times more active than *ortho*, or *meta*, isomers. Greater differences than this would hardly place a compound in the same class as the highly active factors, or alternatively, would lead one to suspect impurities in the test substance.



Certain chemical types acting as growth factors might yield *p*-aminobenzoic acid through a series of simple chemical changes. For instance, compounds 4, 5, 6, 7, 8, 9 in Table 2 are readily changed to *p*-aminobenzoic acid either by hydrolysis, reduction, oxidation or a combination of these reactions. Since these types of reactions characterize many phases of bacterial metabolism, there is good reason for regarding *p*-aminobenzoic acid as the key factor into which other molecules are converted. Certain observations give strong support to this contention. Cultures of the bacterium are capable of hydrolysing Novocaine (7, Table 2) into a primary aromatic amine which, on diazotization, gives a red colour characteristic of *p*-aminobenzoic acid. Furthermore, it was also demonstrated that *p*-nitrobenzoic acid (8, Table 2) was reduced to a similar product. In view of this experimental evidence the following series of reactions are tentatively suggested to account for the growth stimulating properties of several factors detailed in Tables 2 and 3.

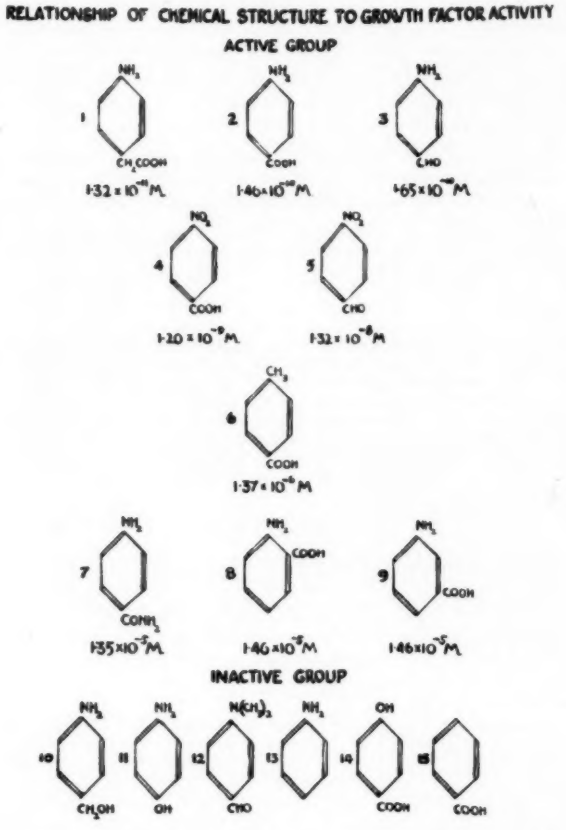
In Table 3 the structural formulae of substances pertinent to this discussion have been drawn. It will be seen that *p*-aminophenylacetic acid (1, Table 3) is the most active factor so far discovered. It is approximately ten times more active than *p-aminobenzoic* acid and, therefore, unlikely to be converted to the simpler acid by de-methylation. In fact, evidence from an entirely different standpoint, namely, the absence of an anti-sulphanilamide action on the part of this compound confirms the view that it functions as a growth factor without any preliminary change in the molecule.

Some general considerations are permissible in regard to the relationship of chemical structure to growth factor activity.

We find that compounds possessing growth stimulating properties are aromatic compounds containing a di-substituted benzene ring in which the substituents occupy 1:4 positions. This orientation is probably of fundamental importance and any departures from it very considerably reduce the activity of the new compound, as is evident when we compare the activity of the aminobenzoic acids. It is even possible that the slight activity of the *ortho* and *meta* acids is due to traces of *para* impurity, in which case the *para* position becomes even more significant. In regard to the type of substituents, it appears that greatest activity is shown when one of the two is an amino group and the other a carboxyl or carboxymethyl group (*vide* subs. 1 and 2, Table 3). Derivatives of these chemical types, or any compounds possessing substituents which, as already indicated, yield amino and carboxyl groups would be included in the above category (*vide* subs. 3, 4, 5, Table 3). Other modifications of the type substance beyond these limits will reduce or completely remove the growth factor properties of the new compound. It is of interest to note that the formation of an amide of *p*-aminobenzoic acid effects a significant reduction in activity (compare subs. 2 and 7, Table 3). This is in striking contrast to the behaviour of nicotinic acid and its amide both of which function equally well as growth factors for *Staph. aureus* and dysenteric organisms. The importance of the amino and carboxyl substituents is best illustrated by noting the formulae of substances in the inactive group of Table 3. Thus, replacement of –COOH in the 1 : 4 di-substituted ring by $-CH_2OH$ or –OH, or the replacement

of NH_2 by $-N(CH_3)_2$ or $-OH$, results in complete inactivation of the new derivative. And finally, mono-substituted benzene derivatives, such as aniline and benzoic acid (subs. 13 and 15, Table 3) are devoid of function.

TABLE 3.



PART III. FERMENTATION OF GLUCOSE IN SYNTHETIC MEDIA BY *CL. ACETOBUTYLICUM* (W.).

In this study the normality of fermentation has been judged by comparison of the yields of solvents formed in the synthetic medium with those obtained in wheat mash containing the same amount of fermentable carbohydrate and incubated for the same period of time. As a result, it has been found that in a *p*-aminobenzoic acid basal medium normal yields of butyl alcohol are formed, but that the production of acetone is suppressed, even after prolonged fermentation. A full explanation of this interesting but "atypical" form of fermentation cannot be given at present but certain observations have been made which offer a partial explanation. It appears that the fermentation of glucose under these conditions

is diverted through the absence in the medium of a second factor, termed *acetone factor*, which is primarily concerned with the formation of acetic acid from acetaldehyde, and secondarily, its conversion to acetone *via* acetoacetic acid. This contention is partly substantiated by the following facts:

1. The *acetone factor* can be isolated from yeast which, on addition to *p*-aminobenzoic acid medium, restores normality of fermentation in respect to yields of solvents.
2. The acetone factor alone does not support growth.
3. Addition of sodium acetate to the synthetic medium does not yield acetone.
4. The titratable acidity in synthetic medium is abnormally low but is raised in the presence of the *acetone factor*.

In view of these findings we consider that two factors are required for normal fermentation of glucose, namely, *p*-aminobenzoic acid acting as a growth stimulant, and the acetone factor which is essential for production of acetone.

Experimental. Sterile 100 ml. volumes of basal medium (page 189), but containing 4 p.c. of glucose, were pipetted into 200 ml. conical flasks. The medium was enriched by the addition of growth factors, extracts, etc., and inoculated with 3·0 ml. of a 24 hour wheat mash culture of the organism. Two cultures, No. 862 A.T.C.C. and Strain F (Australian), were used for all experiments. Analyses of the fermentations were made after 72 hours incubation anaerobically at 37° C. Acetone was estimated according to the method of Goodwin (1920) and ethyl and butyl alcohols by Johnson's method (1932). The analytical results are recorded in Table 4.

The yeast extracts from stages I, II and III contained both factors as indicated by the solvents formed in experiments 2, 3 and 4. Yeast extract of stage V, however, was fractionated by phosphotungstic acid precipitation (page 189) into two portions, one containing the growth factor, experiment 5, and the other containing the *acetone factor*, experiment 6. Whilst in the latter experiment no growth took place due to the absence of growth factor the fermentation was restored to normal by combining the two fractions VA and VB, experiment 7. Evidence of the inability of *p*-aminobenzoic acid to provide a full nutritional background for fermentation is seen in experiment 8.

TABLE 4.

Fermentation of glucose in synthetic media by Cl. acetobutylicum (*W.*) *after 72 hours incubation at 37° C.*

Exp.	Basal medium containing per 100 ml.	Yields calculated as p.c. glucose present. Butyl alcohol.	Acetone.	Comment.
1.	Control: 6 p.c. wheat mash	21·4	10·4	Normal
2.	1 ml. Yeast extract: Stage I	20·2	9·9	„
3.	1 ml. „ „ Stage II	19·8	9·5	„
4.	1 ml. „ „ Stage III	19·7	9·6	„
5.	1 ml. „ „ Stage VA	20·3	Nil	*Acetone factor* removed. Atypical.
6.	1 ml. „ „ Stage VB	0	0	*Acetone factor* only.
7.	1 ml. Stage VA, 1 ml. Stage VB	20·1	8·9	Normal
8.	0·10 μ gm. *p*-aminobenzoic acid	21·2	Nil	Atypical.
	Medium as in Exp. 8 containing per 100 ml.			
9.	1 ml. yeast extract: Stage VB	20·5	9·1	Normal.
10.	1 ml. potato juice	20·1	9·8	„
11.	0·10 gm. gluten	20·3	Nil	Atypical.
12.	0·15 gm. casein	21·4	Nil	„
13.	0·50 gm. sodium acetate	20·9	Nil	„
14.	10·00 μ gm. of each of: thiamin, inosite, tryptophane, cysteine, nicotinic acid, ascorbic acid, pimelic acid	20·5	Nil	„
	No growth 0.			

Note. Potato juice was prepared by expressing 10 ml. juice from 50 gm. of potato and autoclaving.

It is worth noting that the several substances available to us could not replace the *acetone factor* in the *p*-aminobenzoic acid medium. For instance, the deficiency is not due to lack of protein nitrogen (experiments 11 and 12), nor to the absence of special amino acids or other known growth factors (experiment 14). Furthermore, the addition of sodium acetate (experiment 13), does not yield acetone which seems to indicate that one alteration in the mechanism of fermentation lies in the inability of the organism to convert acetic acid to acetone. Finally (experiment 10), it will be seen that potato juice completely fulfils the requirements of the organism.

As regards the chemical nature of the acetone factor little can be said. It is a heat stable acid-resistant factor, probably a co-enzyme, incapable of supporting growth, and present in yeast, cereals and potatoes. It appears to be a nitrogenous base soluble in butyl alcohol and precipitated by phosphotungstic acid. In some earlier experiments on the isolation of the growth factor crystalline flavianate and picrate derivatives were recovered at stage VB, the fraction now known to contain the *acetone factor*. These were derivatives of a basic nitrogenous compound giving a weakly positive ninhydrin reaction. The melting point of the flavianate was 236° C. with decomposition. Unfortunately at the time the *acetone factor* was unsuspected so that there is no experimental evidence to prove the identity of the *acetone factor* with these derivatives. Nevertheless, the finding is suggestive of this idea.

In an attempt to gain further information on the mode of action of the *acetone factor* the titratable acidity during fermentation was determined and compared with that of a normal wheat mash fermentation. To this end the amount of 0·1 N. NaOH required to neutralize 10·0 ml. of metabolism solution was determined at 24 hour intervals over a period of 5 days. The results are best expressed graphically.

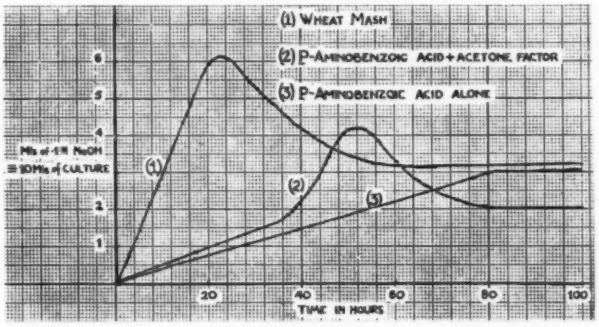


Fig. 5.

It will be seen that the acidity in the synthetic media, curves 2 and 3, does not reach the level attained in wheat mash, curve 1, although curve 2, in which medium normal yields of both solvents are obtained, approximates in type to the normal. However, in the case of the *p*-aminobenzoic acid medium the acidity rises slowly to a maximum of 3·0 ml. ·1 N NaOH after 80 hours incubation.

These results suggest that the organism, in the *p*-aminobenzoic acid medium, fails to synthesize sufficient acetic acid from which acetone might be produced. It will be remembered also (experiment 13, Table 4) that had acetic acid been formed in quantity the yields of acetone would still be negative. Thus the *acetone factor* plays a dual role, being concerned with the formation of acetic acid intermediate and with the conversion of this acid into acetone.

PART IV. FERMENTATION OF WHEAT MASH BY *CL. ACETOBUTYLICUM* (W.).

The solvent production of nine strains of *Cl. acetobutylicum* (W.) was estimated with the view of confirming the identity of the Australian strains with known cultures from America and also, to determine the suitability of Australian wheat for fermentation. In addition, numerous procedures were carried out in an attempt to increase the yields of solvents or their rate of production in industrial wheat mash medium.

Fig. 6 has been constructed from the mean figures of many analyses made at 12 hour intervals during fermentations with several strains of the bacterium. The fermentations were conducted at 37° C. in anaerobic flasks containing 2·5 litres of 6 p.c. wheat mash. The acidity figures, as before, refer to the number of ml. of 0·1 NaOH required to neutralize 10 ml. of metabolism solution.

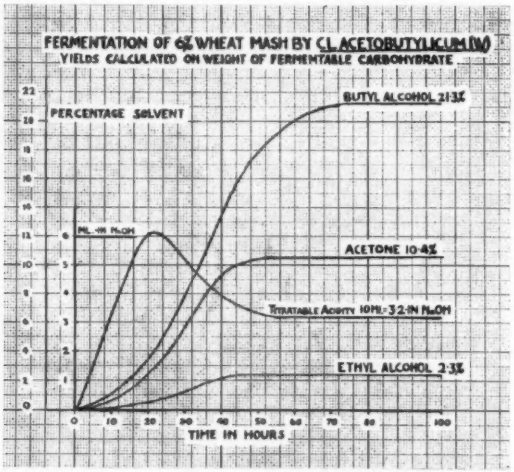

FERMENTATION OF 6% WHEAT MASH BY CL. ACETOBUTYLICUM (W)
YIELDS CALCULATED ON WEIGHT OF FERMENTABLE CARBOHYDRATE
PERCENTAGE SOLVENT
BUTYL ALCOHOL 21·3%
ACETONE 10·4%
TITRATABLE ACIDITY 10 ML. = 3·2 IN N/10 NaOH
ETHYL ALCOHOL 2·3%
TIME IN HOURS

Fig. 6.

It will be seen that a high acidity is built up in the first 24 hours during which solvent production is negligible. As the acidity falls steeply during the 24–48 hour period the solvents rapidly accumulate, acetone reaching a maximum at the 55 hour period, whilst the butyl alcohol maximum is reached approximately 10 hours later. The acidity curve is of value in following the fermentation for, in the event of contamination or "sickness" of the culture, the acidity is maintained at an elevated level.

TABLE 5.

Fermentation of wheat mash by Cl. acetobutylicum (*W.*) *after 72 hours incubation at 37° C.*

Exp.	6 p.c. wheat mash containing	Titratable acidity ml. 0·1 N NaOH.	Yields calculated as percentage of total starch present. Butyl alcohol.	Acetone.	Ethyl alcohol.	Total solvents.
1.	Wheat mash 2·5 p.c.	2·92	21·60	11·10	2·60	35·30
2.	„ „ 5·0 p.c.	2·90	20·80	10·70	2·50	34·00
3.	„ „ 6·0 p.c.	2·86	20·70	10·50	2·50	33·70
4.	Sterilized at 115° C. ½ hr.	2·98	20·40	10·50	2·45	33·35
5.	„ „ 115° C. 1 hr.	3·12	20·00	10·20	2·45	32·65
6.	„ „ 115° C. 2 hrs.	3·88	18·80	9·80	1·64	30·24
7.	+ *p*-aminobenzoic acid 0·01 μ gm. p.c.	3·10	21·45	10·02	2·2	33·67
8.	+ yeast extract Stage I	2·93	20·80	10·40	2·63	33·83
9.	+ $CuSO_4$, $5H_2O$ 10^{-6} conc.	2·92	20·60	10·40	2·45	33·45
10.	„ „ 10^{-5} „	4·35	—	—	—	—
11.	„ „ 10^{-4} „	0	0	0	0	0
12.	+ $FeSo_4$, $7H_2O$ 10^{-6} „	2·96	20·80	10·60	2·56	33·86
13.	„ „ 10^{-5} „	2·92	21·00	10·10	1·97	33·07
14.	„ „ 10^{-4} „	2·87	20·80	11·40	2·66	33·86
15.	+ $NH_4H_2PO_4$ 10^{-2} M. „	4·04	20·20	10·50	2·42	33·12
16.	+ Na_2HPO_4, 10^{-2} M. „	4·01	20·30	10·10	2·74	33·14
17.	Thioglycollic acid 10^{-3} „	2·80	20·50	10·70	2·42	33·62
18.	Ascorbic acid 10^{-3} „	2·80	20·50	10·40	2·70	33·60
19.	Continuous agitation	2·92	20·50	10·20	2·58	33·28

Not estimated —. No growth 0.

The pigmented clostridia described on page 187 conform to this graph whilst cultures of *Granulobacter pectinovorum* A.T.C.C. yielded only traces of solvents. It was evident that these latter cultures could not be classed as butyl alcohol-acetone fermenters.

In Table 5 we have recorded the results of many modifications of the standard wheat mash fermentation. It was hoped that some beneficial effect might be discovered but, as will be seen, none of these procedures increased the yields of solvents.

The conclusion to be drawn from these results is that wheat contains all the specific nutritive requirements, phosphates and reducing substances necessary for optimal growth and fermentation. On the other hand, overheating of the wheat mash and traces of copper in solution reduce the efficiency of fermentation. These facts should be borne in mind in the planning of a large scale industrial fermentation.

SUMMARY.

The systematic bacteriology of *Cl. acetobutylicum* (W.) is described. Features distinguishing this organism are the palisade arrangement of cells in young liquid cultures, vigorous growth in wheat mash with the formation of solvents, growth on a selective *wheat extract agar* medium with the development of circular, creamy white, viscid colonies, the inability to grow in broth or on blood agar plates and the specific nature of the "O" antigen.

The bacterium requires a factor for growth conforming to a structural pattern of a di-substituted benzene ring in which the substituents occupy the 1:4 positions. Optimal activity is developed when the substituents are an amino group on the one hand and a carboxyl or carboxymethyl on the other. Thus, *p*-aminobenzoic acid stimulates growth in concentrations of $1{\cdot}46 \times 10^{-10}$M. and *p*-aminophenylacetic acid in $1{\cdot}32 \times 10^{-11}$M. concentration. The method of isolation of *p*-aminobenzoic acid from brewer's yeast is described. Substances yielding *p*-aminobenzoic acid by hydrolysis, reduction, oxidation, or combination of these reactions, function as growth factor for the bacterium. Spores possess the same requirements as vegetative forms in this respect.

No acetone is produced during fermentation of glucose in synthetic medium containing *p*-aminobenzoic acid. Normal yields of acetone are formed on the addition of a second factor, *acetone factor*, to the medium. This factor does not support growth, is heat stable, precipitated by phosphotungstic acid, and present in yeast, cereals and potatoes. It is probably a co-enzyme and related to the nitrogenous bases. Its mode of action is concerned with the formation of acetic acid from acetaldehyde and the conversion of this acid to acetone through acetoacetic acid as intermediate.

Fermentation of wheat mash is illustrated by a graph in which it is shown that 21 p.c. butyl alcohol, 10·5 p.c. acetone, and 2·5 p.c. ethyl alcohol are formed (calculations based on total starch available). Various modifications of wheat mash fermentation do not increase yields of solvents or their rate of production. On the other hand, traces of copper in solution and prolonged sterilization of the wheat mash were found to be inimical to fermentation.

Acknowledgments. Our thanks are due to Professor H. A. Woodruff for facilitating this work, to the Council for Scientific and Industrial Research for a grant to one of us (M.M.), to Associate-Professor Davies for suggesting the use of *p*-aminophenylacetic acid, and supplying us with this acid and other compounds, to Dr. Adrien Albert for certain chemicals, to Dr. A. W. Turner for photomicrographs (Figs. 1, 2, 4) and Mr. K. Jones (Fig. 3).

REFERENCES.

Bergey: Manual of Determinative Bacteriology, 5th Ed., 1939, London, Balliere, Tindall and Cox.
Brown, R. W., Wood, H. G. and Werkmann, C. H. (1938): J. Bact., 35, p. 206.
Brown, R. W., Wood, H. G. and Werkmann, C. H. (1939): *Ibid.*, 38, p. 631.
Goodwin, L. F. (1920): J. Amer. Chem. Soc., 42, p. 39.
Johnson, M. J. (1932): Ind. Eng. Chem., Anal. Ed., 4, p. 20.
McCoy, E., Fred, E. B., Peterson, W. H. and Hastings, E. G. (1926): J. Infect. Dis., 39, p. 457.
McCoy, E. and McClung, L. S. (1935): *Ibid.*, 56, p. 333.
Oxford, A. E., Lampen, J. O. and Peterson, W. H. (1940): Biochem. J., 34, p. 1588.
Rubbo, S. D. and Gillespie, J. M. (1940): Nature, 146, p. 838.
Rubbo, S. D. and Gillespie, J. M. (1941): Lancet, in press.
Weizmann, C. and Rosenfeld, B. (1937): Biochem. J., 31, p. 619.
Weizmann, C. and Rosenfeld, B. (1939): *Ibid.*, 33, p. 1376.
Weyer, E. R. and Rettger, L. F. (1927): J. Bact., 14, p. 399.
Woods, D. D. (1940): Brit. J. exp. Path., 21, p. 74.

THE REACTION OF MUSCLE TO DENERVATION IN COLD BLOODED ANIMALS

by G. REID[1]

(From the Department of Physiology, the University of Melbourne).

(Accepted for publication 25th June, 1941.)

Several reports (Langley, 1908, 1916), Scaffidi (1913), Watts (1924), Bremer and Gérard (1925, 1926), Tower (1939) have indicated that the denervated muscle of cold blooded animals does not exhibit those morphological and functional changes which are so characteristic of denervated mammalian muscle. These features of denervated mammalian muscle consist, briefly, of atrophy of the muscle fibres, alteration in number, size, shape and disposition of the subsarcolemmal nuclei, fibrillation and alteration in excitability (Tower, 1939). On the other hand Hofmann in 1894 confirmed Erb's (1868) observations that the denervated frog's muscle showed a considerable decrease in excitability to the faradic current. Hofmann also described morphological changes. Apostolaki and Dériaud (1925) described a four- or five-fold increase in chronaxie of the frog's gastrocnemius 4 months after nerve section. More recently, Holobut and Jalowy (1936) showed that the characteristic changes in chronaxie of muscle after denervation appeared much less constantly and much later in frogs than in rats. Frogs were examined up to 149 days after nerve section but in the majority of animals regeneration had already taken place after 69 days.

Some investigators have kept animals for a much shorter period, e.g. Watts (1924) up to 44 days. Practically all the investigations have been made on frogs and except for a few of Langley's (1908) experiments the hind limbs only have been used. Again, many observers do not state whether winter or summer frogs were used.

This report presents the results of experimental denervation in several types of cold-blooded animals.

MATERIALS AND METHODS.

Four species of animals were used in this investigation: the frog (*Hyla aurea*), the common toad (*Lymnodynastes dorsalis*), a large toad (*Bufo marinus*) and the tortoise.

The animals were operated upon under ether anaesthesia with aseptic precautions. In the frogs and toads either the right sciatic nerve or the left brachial plexus was divided, the former in the upper part of the thigh, the latter (through an incision in the axillary region), at the level of the tips of the vertebral transverse processes. In the tortoise the right sciatic nerve was divided. All the animals were kept in concrete tanks portion of which contained water. An earth plot in which grass was sown was incorporated in the tank. There was a considerable mortality particularly among the frogs (about 40 p.c.). Death usually occurred within the first month and was due, as a rule, to wound infection. The frogs and common toads which were kept over two months were fed twice a week by inserting small pieces of meat into their mouths. Despite this there was considerable general wasting in these animals. No attempt was made to feed the large toads or the tortoises. The large toads were very satisfactory subjects and thrived up to six months. Ulceration of the denervated limb was uncommon in any of the animals.

The gastrocnemius or triceps muscles of the frogs and toads which survived three weeks or longer were weighed in the fresh state, and in some cases again after drying to constant weight in a hot air oven at 60° C. Muscles were weighed either at death or after the animals were killed.

At intervals from 20–189 days after denervation selected animals were pithed (brain and cord) and the muscles examined for macroscopic alteration, in particular for the presence of

[1] This research was carried out with the aid of technical assistance provided by the National Health and Medical Research Council.

fibrillation. Electrical reactions were determined on each side for the triceps and flexor carpi radialis muscles in the upper limb, the gastrocnemius and sometimes the peroneal muscles in the lower limb. In the tortoise the flexor digitorum longus muscle was examined. The tests were performed on the exposed muscles *in situ*. The excitability of these muscles to the faradic current was tested by means of an induction coil using either single break stimuli or repeated stimuli. The intensity of stimulation which just caused a response visible with the naked eye, was expressed in terms of the distance between the primary and secondary coils; brass electrodes ·5 cm. apart were used. Strength-duration curves were plotted by means of condensers charged at varying voltages through a potentiometer and discharging through a resistance of 11,000 ohms. The capacity of the condensers ranged from 20 to ·001 microfarads and for convenience the duration of current flow has been expressed in terms of microfarads, the resistance being constant. The cathode was applied to the muscle and the anode consisted of a plate either inserted in the animal's mouth or in the abdominal cavity. In the early experiments the cathode was a stigmatic silver-silver chloride electrode applied to the muscle and the anode was a silver plate coated with silver chloride. Subsequently a simple brass stigmatic cathode applied to the muscle and a brass plate for the anode proved equally satisfactory. The presence or absence of response was determined with the naked eye.

Corresponding muscles (triceps, flexor carpi radialis or gastrocnemius) from each side of several of the animals were examined histologically after fixation in 10 p.c. formalin and staining with iron haematoxylin. In a few experiments the diameters of 100 muscle fibres from corresponding muscles were examined in teased gold chloride preparations. The confirmation of nerve degeneration was established after the animals were pithed, by stimulation of the cut stump of the nerve and, also, in many cases by examining the motor end-plates after treatment by formic acid and gold chloride (Ranvier). The weights of the muscles of a number of normal toads and frogs were determined.

In those cases where Student's "t" test was used the varying periods of denervation in the particular group under consideration were disregarded, the animals for the purpose of the test being regarded as a homogeneous group.

RESULTS.

Morphological changes. Of 60 frogs, in each of which the brachial plexus was divided in March, April or May, 1940, 37 survived for periods ranging from 28–145 days. Of these, 9 survived from 66 to 145 days. The triceps weight of these 9 are set out in Table 1. It can be seen that the maximum percentage decrease in weight is only 13 p.c. in a frog 90 days after denervation. A difference of this order occurred in 3 out of 36 normal frogs. The mean difference between the weights of the triceps (right minus left) for 36 normal frogs was 1 mg. whereas that for the 9 frogs after two or more months denervation is 5 mg. This is not a significant difference ("t" test).

TABLE 1.

Weight in mg. of triceps of frogs after two or more months denervation.

Duration of denervation in days.	Normal.	Denervated.	Difference.	Percentage difference.
65	69	74	— 5	— 7·2
62	75	74	1	1·3
61	105	91	14	13·3
65	126	121	5	4·6
90	31	33	— 2	— 6·5
90	137	119	18	13·1
96	75	67	8	10·6
100	61	68	— 7	— 8·9
145	147	134	13	8·6

Mean difference = 5·0 mg. Standard error of mean = 3·0. "t" = 1·7.

Nine frogs whose right sciatic nerves were divided in November, 1940, survived periods from 39 to 67 days. The weights of the gastrocnemii muscles from these animals are set out in Table 2. Here again atrophy, as represented by loss of weight, is not a striking feature of denervation although in one frog a 34 p.c. decrease is recorded. The mean difference (weight of normal side minus weight of operated side) is 23·3 mg. again not very significant on application of the "t" test. The probability of such a result occurring by chance alone lies between 1 in 10 and 1 in 20.

Of 5 toads (*Lymnodynastes*) whose right sciatic nerves were divided in July, 1940, not one showed any atrophy up to 89 days.

TABLE 2.

Weight in mg. of gastrocnemii of frogs after denervation of the right side.

Period of denervation in days.	Left gastrocnemius.	Right gastrocnemius.	Difference left–right.	Percentage difference.
39	126	106	20	16·0
39	138	137	1	0·7
39	222	200	22	9·9
47	187	167	20	10·7
50	222	260	— 38	— 17·2
50	342	322	20	5·8
54	311	240	38	32·2
52	118	80	71	22·8
67	286	230	56	19·5

Mean difference = 23·3 mg. Standard error of the mean = 10·41. "t" = 2·24

The weights of the triceps or gastrocnemius muscles of 13 large toads (*Bufo marinus*) operated on in October, 1940, and examined 20–189 days after operation are set out in Table 3. Here again atrophy is not a striking feature even up to 189 days after denervation, although in the case of the gastrocnemii the difference between the two sides is just statistically significant.

No further significant results were obtained by re-weighing the muscles after drying to constant weight. In several of the amphibians the denervated muscles were distinctly paler in colour than those on the normal side.

TABLE 3.

Weights in mg. of gastrocnemii or triceps muscles of toads (Bufo marinus) *after denervation.*

Duration of denervation in days.	Normal side.	Operated side.	Difference from normal.	Percentage difference.
Triceps	mg.	mg.		
35	572	629	— 57	— 10·0
95	343	330	13	3·8
97	365	355	10	2·7
189	330	290	40	12·1
189	272	244	28	10·3
189	232	217	15	6·5
189	384	388	— 4	— 1·0
Mean difference = 6·4 mg. Standard error of the mean = 11·6. "t" = 0·55.				
Gastrocnemii	mg.	mg.		
20	525	470	55	10·5
34	699	716	— 17	— 2·4
46	610	561	49	8·2
95	487	432	55	11·9
189	472	380	92	19·5
189	241	225	16	6·6
Mean difference = 41·7 mg. Standard error of the mean = 15·3. "t" = 2·73.				

Usually, significant microscopic alterations in the denervated side did not occur. Generally speaking there was less definite striation on the affected side after several weeks of denervation but all parts of the muscle were not equally affected nor was striation on the sound side always a prominent feature in the sections obtained.

Measurements made of 100 consecutive fibres in teased gold chloride preparations revealed at the most only slight atrophy. Thus in one frog 90 days after division of the left brachial plexus, the ratio of fibre size in the right and left flexor carpi radialis muscles was as 16: 15.

In two out of 45 frogs, however, there were more marked changes in the extrafusal muscle fibres resembling some of the changes met with in mammalian muscle after denervation.

Fig. 1A and B are from the triceps muscles of a winter frog killed 100 days after denervation. Changes on the denervated side consist of increase in number, enlargement and arrangement in parallel rows, of the nuclei, most of which contain one central nucleolus. In transverse section many of these are in the depths of the fibre. Transverse striation is still present. There is marked cellular infiltration in the interstitial tissue. The "chronaxie" values of these muscles were 0·03 microfarads for the normal and 0·13 for the denervated side.

Fig. 1A.

Fig. 1B.

Fig. 1A is from the left triceps of a frog 100 days after division of the left brachial plexus. The increase in number of nuclei and alteration in their shape, pattern and disposition are striking. There is also marked interstitial infiltration of small round cells. × 360.

Fig. 1B is from the normal side. × 360.

Fig. 2A.

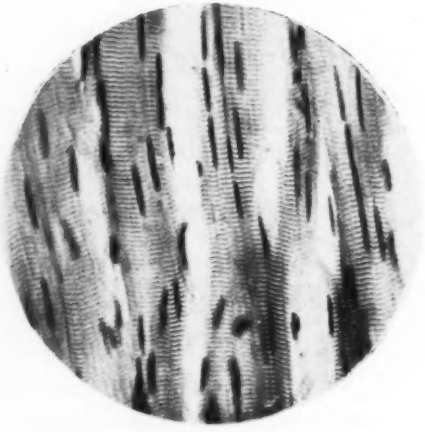


Fig. 2B.

Fig. 2A is from the right gastrocnemius of a frog 39 days after division of the right sciatic nerve. The alteration in muscle outline and structure, with faded or absent transverse striations and increase in size, number and altered orientation of the nuclei are shown. × 360.

Fig. 2B is from the normal side. × 360.

Fig. 2A and B are from the gastrocnemius muscles of a summer frog killed 39 days after denervation. On the denervated side the chief feature was a patchy infiltration of cells in the intercellular tissue. As a rule the striation was preserved in the muscle fibres but in the area shown there was loss of the normal muscle outline with patchy staining of the cytoplasm, enlargement and increase in number of subsarcolemmal nuclei and irregularity in their disposition. The "chronaxie" values of the normal and denervated sides were ·03 and ·09 microfarads respectively. The denervated muscle showed fibrillation.

No morphological changes were met with in any of 20 tortoises up to 149 days.

Fibrillation. In not one out of ten frogs observed between April and October, 1940 (28–145 days) was fibrillation observed in the denervated fore-limb; but in six out of six frogs between November, 1940, and January, 1941 (39–67 days) it was seen in the denervated hind limb. Spontaneous fibrillation was seen in one toad (*Lymnodynastes*) in October, 1940, 89 days after division of the right sciatic nerve. Of the large toads (*Bufo marinus*) operated on in October, 1940, 4 animals from 20–46 days after denervation showed no fibrillation, but 9 out of 9, from 95 to 189 days showed fibrillation either in the fore or hind limb. It was not seen up to 149 days in any of 6 tortoises, operated on in August, 1940.

In all cases in which fibrillation was observed it could be seen in all the muscles of the region of denervation. In most of the animals it was spontaneous, in the others it could be elicited following mechanical or electrical stimulation or warming of the muscle and continued for about a minute or persisted indefinitely. The rate varied from 60 to 100 beats per minute in any one particular point and could be increased by pouring warmed Ringer's solution over the muscle in question. Thus in one frog the rate of fibrillation in the right gastrocnemius at 18° C. increased from 100 to 140 on pouring Ringer's solution at 25° C. over the muscle.

Changes in excitability. In every animal examined there was diminished excitability to the faradic current on the denervated side. This was found even in animals whose strength-duration curve for the muscle in question was unaltered. In all species of animals it was found as early as three weeks. Tests were not made earlier than this.

In a typical experiment with the tortoise 149 days after denervation using a single break stimulus, the minimal intensity necessary to produce a contraction was obtained with the secondary coil at 12 cm. for the normal side and at 6·5 cm. for the denervated side.

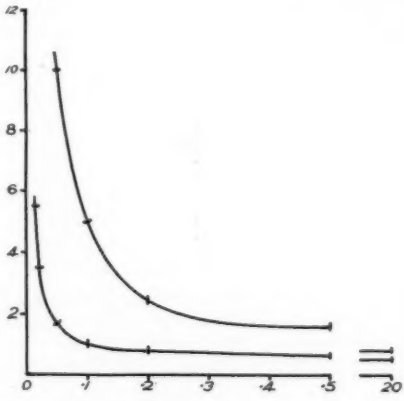


Fig. 3.

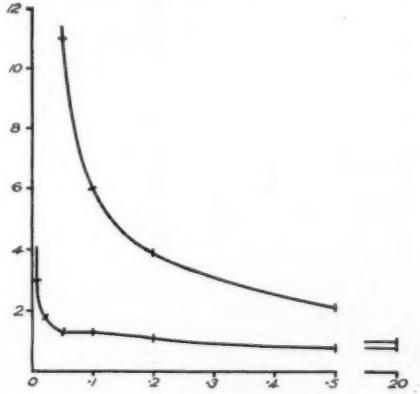


Fig. 4.

Fig. 3. Strength-duration curves of gastrocnemius muscles of a large toad (*Bufo marinus*) 95 days after division of the right sciatic nerve.

Fig. 4. Strength-duration curves of flexor digitorum longus muscles of a tortoise 136 days after division of the right sciatic nerve.

Ordinates in volts. Abscissae in microfarads. The upper curves are from the denervated sides.

Strength-duration curves. Strength-duration curves were drawn of corresponding muscles from a number of non-denervated frogs (10), large toads (10) and tortoises (10). The differences between innervated and denervated muscles described below are in every case outside the normal limits of difference variation in the control groups. Occasionally composite curves showing the α and γ forms of Lucas were obtained (Fig. 4 lower curve) on the innervated muscle but in such cases neither portion of the curve resembled the curve of the corresponding denervated muscle. Definite alteration in the shape of the strength-duration curves with increase of chronaxie on the denervated side was obtained, in the case of frogs, in 5 out of 7 experiments in the fore limb and 6 out of 6 experiments on the hind limb. In the toads (*Bufo marinus*) alterations in the strength-duration curves were seen in 4 of 6 hind limb experiments and 6 out of 7 fore limb experiments. Changes were not always found in every muscle examined in a given region. Sometimes the triceps only, sometimes the flexor carpi radialis only but more usually both, showed typical alterations in the strength-duration curve.

TABLE 4.

"Chronaxie" values in thousandths of microfarads.

Animal.	Duration of denervation in days.	Muscle.	Chronaxie. Normal.	Chronaxie. Operated.
Winter frogs	31	Triceps	80	80
	45	Triceps	20	44
	90	Triceps	31	1,000
	90	Triceps	25	25
	96	Flex. carp. rad.	20	24
	100	Triceps	30	130
	145	Flex. carp. rad.	15	200
Summer frogs	39	Gastrocnemius	45	300
	39	"	50	1,000
	39	"	30	900
	47	"	20	200
	50	"	200	200
	67	"	45	300
Summer toads (*Bufo*)	20	Gastrocnemius	60	40
	34	"	60	25
	35	Flex. carp. rad.	30	600
	46	Gastrocnemius	40	25
	95	Flex. carp. rad.	35	200
	95	Gastrocnemius	100	500
	97	Flex. carp. rad.	250	1,000
	189	Flex. carp. rad.	30	400
	189	Flex. carp. rad.	30	Very long
	189	Flex. carp. rad	250	300
	189	Gastrocnemius	20	800
	189	Gastrocnemius	30	600
Tortoises	65	Flexor digit long.	50	800
	80	" " "	70	30
	87	" " "	75	340
	87	" " "	30	600
	149	" " "	50	600
	149	" " "	400	600

All these changes in strength-duration curves were seen between 35 and 145 days in the frogs and 39 and 189 days in the large toads. In none of the common toads was alteration in the strength-duration curves observed (up to 89 days). Changes in the shape of the strength-duration curve were also observed in 4 out of 6 tortoises between 65 and 149 days of denervation. The "chronaxie" values of some of the muscles examined are set out in Table 4. The rheobase was sometimes raised, sometimes lowered, sometimes relatively little altered. Characteristic curves from two experiments are illustrated in Figs. 3 and 4. During stimulation the response on the denervated side was often seen to be localized to the region of the stimulating electrode and sometimes the propagation of contraction through the muscle was sufficiently slow for it to be followed as a slow wave of contraction with the naked eye. These results were never seen on the normal side.

DISCUSSION.

The results just described are consistent with the view that morphological changes are not a conspicuous feature of the denervated muscle of cold-blooded animals, at any rate not up to 5 or 6 months after denervation. The morphological changes illustrated in the figures are exceptional. On the other hand fibrillation and changes in excitability may be definite features although occurring less constantly and at a later period than obtains with mammalian muscle. Loss of weight is not pronounced, although in one frog a loss of 34 p.c. after 52 days was recorded. It is interesting that Langley who failed to find significant loss of weight in his frog's muscles after denervation does mention (1916) that he found one animal with a considerable decrease, 32 p.c., after 95 days. It is possible that loss of weight of this magnitude might be met with more regularly after much longer periods of denervation.

The absence of pronounced atrophy up to 6 months after denervation is possibly related to the temperature difference between these animals and mammals. Bremer and Gérard (1925) claim to have excluded the environment-temperature factor in this connection, but their observations only extend to 42 days. Langley (1916) suggested that the absence of atrophy might be correlated with the absence of fibrillation.

The motor end plates of these animals are considerably different from those of mammals, being more diffuse and widely arborizing (see Hinsey, 1934, for literature), and they react also to acetylcholine directly applied (Sommerkamp, 1928; Wachholder and Ledebur, 1930). This may suggest that their functions are less closely integrated with the action of the nerves supplying them than are mammalian muscles. This morphological and functional difference might perhaps be correlated with their continued existence without atrophy after denervation. On the other hand even if this were true, some atrophy due to disuse might be expected. In point of fact the degeneration of the nerves tissue itself proceeds very slowly in the cold blooded animals. Thus Hofmann (1894) found indirect excitability of the muscle of the frog *(Rana esculenta)* to persist after denervation up to 15–25 days and other later observers have found similar results. Tiegs (1932) also, in his degeneration experiments in the lizard found it necessary to wait several months after denervation for degeneration to be complete.

So far as fibrillation is concerned it is interesting to note that Langley failed to find it in the frog, whereas in these experiments it was a very definite feature in some of the amphibians. It is possible that the failure of other observers to see fibrillation lies in the fact that the muscles were examined in the lower temperatures of Northern European countries or that the animals were not kept for sufficiently long periods, or that seasonal influences may have affected the results. It is perhaps significant that fibrillation was never observed in the winter frogs even up to 145 days, whereas it was found in the summer frogs as early as 39 days and in all the toads examined in summer but not before at least 46 days. In some cases as described, fibrillation though not spontaneous would appear after stimulation of the muscle. This is probably a similar phenomenon to that recently described by Eccles (1941) for mammalian muscle.

Langley (1916) stated that Hofmann found fibrillation in the toad but the reference given is in error and I have not been able to find his paper.

The alterations in the excitability to the faradic current and the changes in the shape of the strength-duration curves were very definite and call for little comment. The exact time of onset of these changes was not determined but change in the faradic excitability was always met with at three weeks, i.e. before change in the strength-duration curve. As is well known, various factors influence the shape of the curve obtained; in particular the size of the stimulating electrode, the interval between the electrodes and their orientation relative to the muscle fibres. For comparison purposes it has been assumed that approximately the same conditions have been obtained on each side. In these experiments on excitability no attempt was made to prevent indirect stimulation of the innervated muscle by means of curare because this might have raised controversial problems. The point at issue arose from the fact that statements have appeared (see Tower, 1939) which indicate that, in cold-blooded animals, no difference has been observed on comparison of the excitabilities of innervated and denervated muscle, using electrodes directly applied to the muscle (which, of course, on the innervated side still contains functioning nervous tissue). These experiments show that such a difference does exist. That the explanation may or may not be that direct stimulation has replaced the indirect stimulation in the denervated muscle is not the immediate concern of this paper. A discussion of this would raise the old controversy over isochronism. At all events in those innervated muscles in which

composite curves were obtained the α or muscle component did not resemble the curve of the corresponding denervated muscle.

Fibrillation was a later development than loss of indirect excitability; for example, fibrillation was never met with in the toad before 46 days whereas loss of indirect excitability was present in less than three weeks. This time relationship roughly parallels the order of onset in mammals, e.g. in cats there is loss of indirect excitability after two days but fibrillation does not appear till the fifth day of denervation (Langley and Kato, 1915).

In conclusion, it can be said that statements in the literature such as that of Bremer and Gérard (1925) that there is "absence complète de dégénérescence (morphologique et functionelle) chez la grenouille, le lézard, la tortue" following denervation are not strictly correct. Morphological changes during the periods of observation are not a marked feature (except in isolated instances) but the fibrillation, the alteration in excitability to faradic stimulation and the change in the strength-duration curves are features of denervation in the muscle of cold-blooded animals as well as of mammals.

Acknowledgments. I am indebted to Professor R. D. Wright for his help and interest in this investigation; to Miss Wischusen, who prepared the photomicrographs; and to the technical staff of the Pathology Department of the University of Melbourne.

SUMMARY.

Morphological changes except in some isolated instances are not a striking feature of denervated muscle in frogs, toads or tortoises.

Fibrillation occurs in denervated amphibian muscle.

Changes in excitability to the faradic current and to condenser discharges are described.

REFERENCES.

Apostolaki, J. et R. Dériaud (1925): C.R. Soc. d. Biol., 92, p. 1482.
Bremer, F. et P. Gérard (1925): *Ibid.*, 92, p. 1327.
Bremer, F. et P. Gérard (1926): *Ibid.*, 94, p. 1035.
Eccles, J. C. (1941): Med. J. Austral., 1, p. 573.
Erb (1868): Deutsches Arch. f. Klin. Med., Bd. 4, p. 535.
Hinsey, J. C. (1934): Physiol. Rev., 15, p. 514.
Hofmann, C. F. (1894): Arch. f. exp. Path. u. Phar., 33, p. 117.
Holobut, W. S. und Jalowy, B. (1936): Z. f. Zellf. f. u. mikr. Anat., 25, p. 541.
Langley, J. N. (1908): J. Physiol., 37, p. 235.
Langley, J. N. (1916): *Ibid.*, 50, p. 335.
Langley, J. N. and Kato, T. (1915): *Ibid.*, 49, p. 410.
Scaffidi, V. (1913): Z. f. Allg. Physiol., 15, p. 329.
Sommerkamp, H. (1928): *Ibid.*, 128, p. 99.
"Student" (1938): Statistical Methods, Snedecor, Iowa.
Tiegs, O. W. (1932): J. Anat., 66, p. 300.
Tower, S. (1939): Physiol, Rev., 19, p. 1.
Wachholder, L. and Ledebur, J. (1930): Pflügers Arch., 225, p. 627.
Watts, C .F. (1924): J. Physiol., 59, p. 143.

A CONTRIBUTION TO THE PHARMACOLOGY OF ADENYL COMPOUNDS, WITH A NOTE ON THEIR OCCURRENCE IN RED BLOOD CELLS.

by IAN ROBERTSON AND F. H. SHAW

(From the Department of Physiology, University of Melbourne).

(Accepted for publication 23rd May, 1941.)

Adenine, adenosine, adenylic acid and adenyl pyrophosphate (A.P.P.) form a series which is playing an ever increasing role in pharmacology, and is an ideal one in which to study the relationship of chemical structure to physiological properties. Earlier work will be found in papers by Drury (1932), Gillespie (1934) and Flossner (1934).

The present contribution is an endeavour to complete certain sequences untouched by previous workers and especially to consider the unusual effect of A.P.P. on the frog's heart.

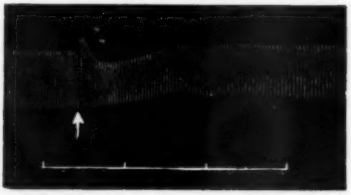


Fig. 1. Frog's heart. Adenyl pyrophosphate 1 in 5,000. Time: half-minutes.

The adenine, adenosine and adenylic acid used in these experiments were obtained from British Drug Houses. The adenyl pyrophosphate was prepared from rabbits' muscle by Lohmann's (1931) method and its constitution was carefully checked by phosphate determinations. The effects of these substances were investigated on the undermentioned test objects.

The Frog's Heart (Straub's method).

Adenine. No action in a concentration of 1: 1,000.

Adenosine. The rate of the heart is markedly slowed, without any effect on the amplitude of the beat, but very often the heart ceases to beat after a short time (1–3 mins.) and remain quiescent in diastole (concentration 1: 10^5).

Adenylic acid. The effect is similar but requires stronger solutions, 1: 10^4.

Adenyl pyrophosphate. There is an immediate increase in amplitude which lasts for about 6 beats, followed by a decrease for about 20 secs., then the amplitude begins to increase again while at the same time a slight diminution in the rate is observed. (Fig. 1.) It should be noted that concentrations of as much as 1: 5,000 are not deleterious, while with adenosine and adenylic acid stillstand may be produced by a concentration as low as 1: 10^6.

Isolated Intestine (Rat or Rabbit).

Adenine. A slight inhibition of rhythmical movement is produced by a dilution of 1: 10^4.

Adenosine and adenylic acid. Cessation of rhythmical movement and relaxation are brought about by a concentration of 1: 10^5.

Adenyl pyrophosphate. In a dilution of 1: 10^4 causes a transitory inhibition of rhythmical contraction followed by an increasing tonus of the musculature. (Fig. 2.)

Perfused Frog's Vessels (Trendelenburg's method).

Adenine. No effect.

Adenosine and adenylic acid. Each brings about an equal state of vaso-dilatation in a dilution of about 1: 10^5.

The Occurrence of a Substance with similar Properties in Red Blood Cells.

Previously one of us (F.H.S.) in an attempt to show pharmacologically the presence of adrenaline in blood, had discovered in erythrocytes, but not in serum, a substance which had an action on the frog's heart similar but not identical with that of A.P.P.

A purification of the cell extract was brought about by an adsorption on aluminium hydroxide at pH 4, at which pH adrenaline is not adsorbed, and then at pH 8·5, when it is adsorbed (see Shaw (1938)). Adenyl compounds behave similarly but may be eliminated by precipitation with silver nitrate.

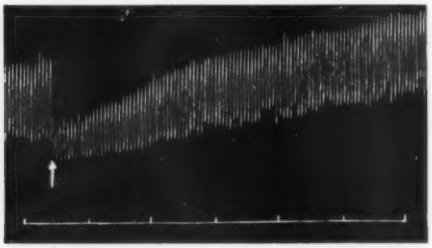


Fig. 2. Rabbit's intestine. Adenyl pyrophosphate 1 in 5,000. Time: half-minutes.

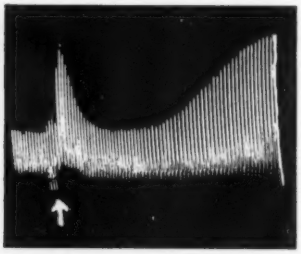


Fig. 3. Frog's heart. Effect of substance isolated from red blood cells. Time: 2 minutes.

To a trichloracetic extract of the red cells of a rabbit or dog were added excess $AgNO_3$ and a drop of dilute nitric acid. The mixture was then centrifuged. Excess silver in the supernatant fluid was removed by the addition of chloride and the double adsorption on colloidal aluminium hydroxide was carried out as described by Shaw (1938). The aluminium hydroxide was diluted and added directly to the bath or cannula. More often the aluminium hydroxide was brought into solution by the addition of 2–3 drops of sodium citrate. The excess citrate reduced the calcium ion content of the Ringer. To overcome this difficulty calcium was added until a control showed that the excess citrate had been "neutralized".

A typical result is shown in Fig. 3. The substance also brought about inhibition of the rabbit's intestine and relaxation of the guinea-pig's intestine.

DISCUSSION.

In agreement with all other workers we find that adenine, even in a strength of 1 : 1000 (saturation point) is practically without pharmacological activity. However, the addition of the pentose brings about considerable increase in activity. Adenosine in concentrations of $1:10^4$–$1:10^6$ is able to affect profoundly the responses of various tissues. The further addition of phosphoric acid only influences quantitatively the new properties now made manifest. On the peripheral blood vessels, duration of heart block in the guinea-pig, and on the coronary vessels of frog's heart adenosine is the more active, on the isolated uterus and intestine adenylic acid has the stronger effect, whilst on the perfused vessels of the frog and of the lung of the cat or dog the effects are about equal. However, the addition of pyrophosphoric acid to the molecule again alters the properties considerably. According to Fleisch (1937) A.P.P. has a strong vaso-dilator action; this is not in agreement with the results of Schoedl (1937), who ranks it as a less powerful dilator than adenosine. It has less action on the coronary vessels than either adenosine or adenylic acid but more on the isolated uterus and on the perfused vessels of the lung. But it is on the frog's heart and the isolated intestine that the most pronounced differences are to be found. Adenosine ($1:10^5$) and adenylic acid bring about a relaxation of the longitudinal muscles of the intestine but A.P.P. ($1:10^4$) causes a rise in tonus. This was first pointed out by Gillespie (1934) for rabbit's intestine and confirmed by us for both rabbit's and rat's intestine. On the frog's heart the nucleoside and nucleotide bring about stillstand in a few minutes, but A.P.P. exerts a most characteristic and to a certain extent beneficial effect. The first to investigate this action were Ostern and Parnas

(1931) and although a perusal of their figures reveals that on some occasions they obtained this characteristic effect they do not appear to have stressed the difference between it and other adenyl compounds. Indeed, in a later paper (Ostern and Parnas (1932)) they say that the frog's heart may be used to assay adenyl compounds; this is true, but the method would be unworkable in the presence of A.P.P. and *vice versa*. The other reference to the action of A.P.P. on Straub's preparation is that of Fleisch (1937), who used this preparation as a qualitative test to show that the vaso-dilator substance which he had isolated from erythrocytes was A.P.P. The effect of this unknown substance on the frog's heart was similar to that obtained by us, but it is unlikely that our substance is A.P.P., as the adenyl compounds were removed by silver precipitation. Also the second increase in the strength of heart beat, obtained both with Fleisch's compound and our own, was not given by pure A.P.P., either in our own experiments or those of Ostern and Parnas (1931). In addition, both unknown substances caused an inhibition of intestinal movement and a relaxation of musculature, whereas, as previously mentioned, A.P.P. brings about an increase in the tonus of this organ. From this we would conclude that neither Fleisch's principle nor our own is A.P.P. Fleisch himself admits that the evidence for the presence of A.P.P. is inconclusive.

Acknowledgment. The authors wish to thank Professor R. D. Wright for providing facilities to enable the work to be carried out and for helpful advice.

REFERENCES.

Drury, A. N. (1932): J. Physiol., 74, p. 147.
Fleisch, A. and Weger, P. (1937): Pflug. Arch., 239, p. 476.
Flossner, O. (1934): Arch. exp. Path. and Pharm., 174, p. 245.
Gillespie, J. H. (1934): J. Physiol., 80, p. 345.
Lohmann, K. (1931): Biochem. Z., 233, p. 460.
Ostern, P. and Parnas, J. K. (1931): *Ibid.*, 234, p. 313.
Ostern, P. and Parnas, J. K. (1932): *Ibid.*, 248, p. 389.
Schoedl, W. (1935): Pflug. Arch., 236, p. 93.
Shaw, F. H. (1938): Biochem. J., 32, p. 19.

THE MECHANISM OF THE PASTEUR EFFECT IN ALCOHOLIC FERMENTATION BY YEAST CELLS

by ALFRED GOTTSCHALK

(From the Walter and Eliza Hall Institute, Melbourne).

(Accepted for publication 16th May, 1941.)

The action of oxygen in checking the high rate of anaerobic carbohydrate destruction and in suppressing or diminishing the accumulation of the products of anaerobic metabolism is called the Pasteur effect, as defined by K. C. Dixon (1937). This effect, observed at first in alcoholic fermentation by yeast (Pasteur, 1861) operates in the same manner in animal cells (Meyerhof, 1920–24; Warburg *et al.*, 1924). The first explanation of the Pasteur effect was offered by Meyerhof (1920). Meyerhof relying on his work on the recovery phase of the isolated frog muscle after electrical stimulation or asphyxia supposed the existence in the muscle of a carbohydrate cycle.

In this cycle the split product, lactic acid, is aerobically formed with the same high velocity as under anaerobic conditions; in a second phase the greater part of the lactic acid is resynthetized to glycogen at the cost of energy supplied by the combustion of a small part of the acid to CO_2 and H_2O. Undoubtedly an oxidative resynthesis takes place in frog muscle if a period of anaerobic glycolysis is followed by a period of oxygenation. The further conclusion, however, that the Pasteur effect is also due to an oxidative resynthesis is hypothetical only (cf. Gottschalk, 1925). Neither in mammalian brain cortex (K. C. Dixon, 1935), in chick embryo (Needham and Dixon, 1937) nor in intact mammalian muscle (Flock and Bollman, 1940) could a synthesis of glycogen from lactic acid be demonstrated though in all these tissues the Pasteur effect is observed.

In the case of alcoholic fermentation by compressed yeast Meyerhof (1925) suggested the existence of a carbohydrate cycle similar to that in muscle. Meyerhof however did not perform a complete carbohydrate balance to demonstrate that there is an increase in the glycogen content of yeast after glucose fermentation under aerobic conditions. The work described in this paper consists of complete determinations of the carbohydrate balance of compressed yeast as well as of the accompanying gaseous exchanges with glucose, fructose and acetaldehyde as substrates under anaerobic and aerobic conditions.

APPARATUS AND METHODS.

The following apparatus (Fig. 1) was designed to permit the complete investigation of the metabolism of the added sugar in addition to the determination of the gaseous exchange. The glass bulb (K), volume = 56 c.c., in which is placed the yeast suspension (15 c.c.), possesses three capillary outlet tubes. The lower tube (a) allows equilibration with various gases, the tube b serves as an outlet during gas equilibration and for the collection of samples for gas analysis. The tube c is connected to a constant pressure gas burette A containing Hg, which allows (1) the increase of the initial gas space above the fluid in K to any desired volume and (2) the measurement of the increase or decrease of the total volume of gas at atmospheric pressure. The bulb K is closed by a rubber stopper containing a thermometer (d) which dips into the yeast suspension. A second thermometer is used to measure the temperature of the surroundings. The bulb K, containing four glass beads, is mechanically shaken at 120–150 oscillations per minute; the levelling bulb e is lifted and lowered every few minutes to ensure a perfect mixing of the gas in K and A; by these means an accurate equilibrium is obtained between the liquid and gaseous phases. At the end of the respiration experiments the gas mixture was analysed for CO_2 and O_2. A sample was driven over and collected over Hg in the glass tube B. 10 p.c. NaOH solution was used for the absorption of CO_2; the oxygen was absorbed by means of a solution containing 9 gm. $Na_2S_2O_4$ in 50 c.c. 10 p.c. NaOH.

The gases used were nitrogen, oxygen and air. The commercial nitrogen was freed from oxygen by allowing it to stand over alkaline $Na_2S_2O_4$ for several days. The commercial oxygen which was used contained 1·5 p.c. inert gas. In the experiments with air the initial total gas space was so adjusted that the final partial pressure of O_2 (at the end of the experiment) was not below 80 mm. Hg which was sufficient in these experiments (temperature: 25° C.) to ensure an adequate oxygen supply. The delivery tubes from the gas containers to the bulb K as well as the bulb K itself were separately evacuated and refilled with the gas several times; 5 litres of the gas were then bubbled through tube a into the yeast suspension and were finally led out through tube b.

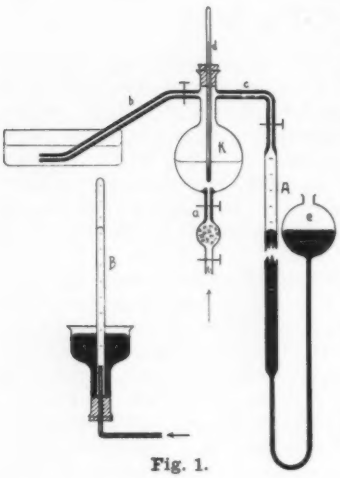


Fig. 1.

The temperature was regulated at 25° C. ± 0·5 C., except where stated.

The burette A could be read to ± 0·05 c.c., which gives an error of 2·5 p.c. in the respiration experiments and less than 0·3 p.c. in the anaerobic experiments. The gas analysis carried out in tube B introduces an error of 1·5 p.c.

The calculations of the gaseous exchange were performed in the following manner:

a. Fermentation in nitrogen. The initial gas volume v'g and the final gas volume v"g were reduced to N.T.P. and corrected for aqueous tension by the formula $vg_0 = vg \frac{T_0 \cdot (b-e)}{T \cdot Po}$ where $T_0 = 273°$; T = absolute temperature in the system, b = atmospheric pressure in mm. Hg, e = maximal tension of the water vapour at T; Po = 760 mm. Hg; vg_0 = vg reduced to normal conditions.

The difference between v"g and v'g equals the CO_2 content in the gas space. The volume v of CO_2 dissolved in the yeast suspension is obtained by the following formula: $v = vf \cdot \alpha CO_2 \cdot xCO_2$, where αCO_2 = absorption coefficient of CO_2 at T, xCO_2 = molecular fraction of CO_2.

b. Fermentation and respiration of the yeast cells under aerobic conditions with glucose (or fructose) as substrate. In the presence of fermentable sugar the respiratory quotient is 1, i.e. respiration CO_2 = O_2 consumption in c.c. (cf. Meyerhof 1925 and this paper, page 219). Therefore the increase (a) measured in the total gas present in the system must be fermentation CO_2. The total amount of CO_2 produced (b) and of oxygen consumed (c) is obtained by analysis of the final gas mixture. The respiratory CO_2 is equal to the difference between total CO_2 and fermentation CO_2. It can be seen from the above notes that the direct oxygen determination by gas analysis serves no further purpose than to act as an additional experimental figure to confirm the other two figures obtained experimentally (c = b–a) and to act as a check on the perfect mixing of the gas in the system and on the satisfactory preliminary equilibration.

c. With acetaldehyde as substrate the experimental procedure is similar. In this case all CO_2 produced and measured is of respiratory origin; the oxygen consumption is obtained from the decrease of the total gas volume (which must be corrected for the CO_2 production) or from the difference between the initial and final oxygen amounts in the gas space as determined by gas analysis. The respiratory quotient is calculated from the experimental values for CO_2 production and O_2 consumption. The small endogenous respiration of the yeast is disregarded because it is highly probable that it ceases in the presence of easily oxidizable substrates (Lundsgaard, 1932; Wieland and Wille, 1933; Stier and Stannard, 1935).

The experimental results were reduced to $Q^{N_2}_{CO_2}$, $Q^{O_2}_{CO_2}$, and Q_{O_2}.

$$Q^{N_2}_{CO_2} = \frac{\text{emm. } CO_2 \text{ produced in nitrogen}}{\text{mg. dry weight of yeast} \times \text{hours}} \quad \begin{matrix}\text{(fermentation under anaerobic conditions} \\ = \text{anaerobic fermentation)}\end{matrix}$$

$$Q^{O_2}_{CO_2} = \frac{\text{emm. fermentation } CO_2 \text{ produced in oxygen}}{\text{mg. dry weight of yeast} \times \text{hours}} \quad \begin{matrix}\text{(fermentation under aerobic conditions} \\ = \text{aerobic fermentation)}\end{matrix}$$

$$Q_{O_2} = \frac{\text{emm. } O_2 \text{ consumed}}{\text{mg. dry weight of yeast} \times \text{hours}} \quad \text{(respiration)}.$$

The number of mg. glucose fermented is calculated from the equation
1 c.c. fermentation CO_2 = 4·02 mg. glucose,
the number of mg. glucose oxidized is calculated from the equation
1 c.c. O_2 consumed (or 1 c.c. respiration CO_2) = 1·34 mg. glucose.

In the experiments with nitrogen a correction was necessary for the decrease in solubility of this gas due to the decrease of the partial pressure of N_2. In the experiments with oxygen or

air the correction was so small that it could be omitted. The vapour pressures of the ethyl alcohol and the acetaldehyde were also negligible in these experiments.

Technique of the Experiments.

The yeast was suspended in M/15 KH_2PO_4 (cf. Krebs, 1928) and pipetted into the bulb K. The pH of the yeast suspension at the beginning of the experiment was 4·3, at the end about 3·3; this made the addition of acid at the end of the experiment unnecessary; this fact was verified several times by the addition of acid and remeasurement of the CO_2. The substrate (glucose, fructose or acetaldehyde) was placed in a small glass container which was suspended in the bulb K by means of a very thin wire pulled through the capillary tubes b and c, the outlet of the former dipping into water. After equilibration with the gas a certain amount of this gas was sucked into the burette A; then for 10 minutes the bulb K was shaken and at the same time the gas in K and A thoroughly mixed to establish thermal equilibration. The wire was then withdrawn under water and the reading of the initial volume taken. At the end of the experiment—in the respiration experiments after collecting a gas sample over Hg—the yeast suspension was quickly transferred into a centrifuge tube which was immediately placed in a thermos flask filled with solid CO_2. After 15 minutes this frozen suspension was centrifuged until it was thawed and the yeast sedimented. By this means autofermentation (if the added sugar was consumed) or fermentation of residual sugar was stopped instantaneously and losses of volatile constituents were prevented.

The experiments were performed with 3 different races of compressed top yeast:

compressed baker's yeast Effront (grown on molasses)
compressed baker's yeast Hudson (grain yeast)
compressed spirit yeast Hudson (grain yeast).

Only quite fresh yeast was used.

The acetaldehyde used was freshly distilled over $CaCO_3$ before each series of experiments and diluted to 2·8 p.c. solution, the exact acetaldehyde content of which was determined before each experiment. After the addition of the acetaldehyde to the yeast suspension the highest initial concentration employed was 0·02 molar. In the experiments I–V of Table 7 half of the acetaldehyde was added at the beginning, the other half after 45 minutes. A determination of the *dry weight* of the yeast was made for each experiment. 0·2 gm. of the fresh yeast, used in the experiment, was weighed into a weighing bottle and suspended in 4–5 c.c. distilled water; the weighing bottle fixed on a wire loop, was put into a beaker with boiling distilled water for 7 minutes and afterwards allowed to dry at 100° C. in an electric oven. In this way autolysis during drying was prevented. In the anaerobic experiments where stress was laid on most accurate figures for the increase in the dry weight during fermentation 5 c.c. of the yeast suspension before the experiment and 5 c.c. of the yeast suspension after fermentation (withdrawn from the bulb K before freezing the content) were dried in the same manner.

The reducing carbohydrates were throughout estimated by the Hagedorn-Jensen micromethod, the results being calculated in all cases as glucose.

The amount of *total carbohydrate* of the yeast cells was determined by heating the yeast, freed from the suspension fluid, for 4 hours with N HCl at 100° C. in a water-bath and determining the reducing substances in the filtrate after neutralization with N NaOH, using 0·1 p.c. phenol red as indicator. The time of 4 hours is necessary for the hydrolysis of any trehalose present in compressed yeast. In control experiments with similar concentrations of pure glucose it was found that heating for 4 hours with N HCl caused 7 p.c. of the added sugar to be lost. To correct for this gradual loss the final figure of total carbohydrate (mg. glucose) was multiplied by 1·03.

Glycogen and gum were determined by digesting the yeast cells in a stoppered centrifuge tube with 60 p.c. KOH for 3 hours in a water-bath at 100° C., diluting with water and making up to 70 p.c. alcohol as described by Mayer, 1923 (cf. Euler, 1914). After several hours in the ice chest the precipitate was separated from the supernatant fluid by centrifugation; it was washed on the centrifuge with 60 p.c. alcohol and absol. alcohol. The dry residue was digested with hot water, the insoluble part separated by centrifugation, digested again with hot water and recentrifuged. A clear solution was thus obtained containing the glycogen and yeast gum, leaving a residue of insoluble carbohydrate. The glycogen-gum fraction was hydrolyzed by heating with N HCl for 2 hours on the water-bath and determined as reducing sugar after neutralization, as recommended by R. A. McAnally and I. Smedley-Maclean (1935).

The insoluble carbohydrate in the residue, still present in the original centrifuge tube, was hydrolyzed and determined in a similar manner. According to McAnally and Smedley-Maclean (1937) this insoluble carbohydrate contains a polysaccharide (pseudoglycogen) which closely resembles glycogen and which gives a quantitative yield of glucose by hydrolysis with N HCl (2 hr. at 100° C.) together with another polysaccharide which when heated with N HCl for 2 hours at 100° C. was attacked liberating glucose and a carbohydrate containing an acid group. The insoluble carbohydrate of the residue is also referred to as membrane polyose, because it is a constituent of the cell membrane.

The acetaldehyde in the standard solution and in the distillate from the suspension fluid of the yeast (*vide infra*) was determined by the hydroxylamine-sulphate method, described by C. Neuberg and A. Gottschalk (1924). In very small aldehyde concentrations this method gives more reliable results than the Ripper method.

For the *determination of the ethyl alcohol* the Widmark micromethod (1922) was used with the following modification. Depending on the amount of alcohol expected 5–12 c.c. of the clear supernatant fluid freed from yeast by centrifugation were distilled over $CaCO_3$, the vapour being condensed in tubes surrounded by ice and collected in an ice cooled receiver. The distillate was made up to 10 or 25 c.c. with water. 0·5 c.c. of this solution was added to 2 c.c. of the biehromate solution, containing 0·250 gm. potassium biehromate in 100 c.c. conc. sulphuric acid and allowed to cool for a few minutes; 50 c.c. cooled water and 1·0 c.c. 5 p.c. potassium iodide solution were added and this final solution titrated with N/100 thiosulphate. The blank determinations were carried out in a corresponding manner. Control experiments using known amounts of freshly distilled absolute ethyl alcohol proved the accuracy of this method. In this modification the quotient $K = \frac{\text{alcohol}}{\text{bichromate}}$ (where "alcohol" is the calculated amount of alcohol in γ, "bichromate" the amount of reduced bichromate expressed in 1/100 c.c.) equals 1·04. Therefore the constant 1·04 was applied to the calculation. In the experiments with acetaldehyde this substance interferes with the alcohol determinations. Therefore in one sample of the distillate the acetaldehyde present was determined by means of $(NH_2.OH)_2.H_2SO_4$. A solution of acetaldehyde of this concentration, prepared from a titrated acetaldehyde standard solution, was submitted to the Widmark determination. A correction, thus obtained, was applied to the results of the Widmark determination of another sample of the same distillate, containing ethyl alcohol besides acetaldehyde.

For the determination of *volatile acids (acetic acid)* the yeast suspension was centrifuged and a part of the supernatant liquid, after acidification, submitted to a steam distillation. The distillate, after the addition of an excess of N/20 NaOH, was titrated with N/20 HCl taking standard precautions for the presence of CO_2. The results of this titration are not very satisfactory in very dilute acid solutions; therefore in the experiments of Table 7 the pH of the distillate was determined by means of the glass electrode and compared with the pH of standard acetic acid solutions.

RESULTS AND DISCUSSION.

Carbohydrate Balance Under Anaerobic Conditions.

It has long been known that in the presence of living yeast more sugar disappears than can be accounted for by the production of ethyl alcohol, carbon dioxide and the small amount of glycerol, the only by-product of alcoholic fermentation referable directly to the sugar (Jodlbauer, 1888; Euler and Johansson, 1912; Euler, 1914; Lundin, 1923; Meyerhof, 1925; Guillemet et Leroux, 1936; Winzler and Baumberger, 1938). In most of these experiments, however, air was present above the yeast suspension so that no definite conclusions concerning the behaviour of the yeast under strictly anaerobic conditions can be drawn from these results. In Meyerhof's experiments carried out under strictly anaerobic conditions only the total amounts of sugar consumed and of CO_2 produced were determined. A complete balance of the anaerobic fermentation of sugar comprising determinations of the CO_2 and alcohol produced and of the fate of the sugar consumed but not fermented has not yet been drawn up. Accordingly the following experiments were performed.

230–750 mg. fresh yeast were allowed to ferment completely about 150 mg. glucose at different temperatures (20°–25° C.). The end of fermentation of the added glucose can be found out accurately. The results of the experiments are shown in Tables 1 and 2 and summarized in Table 3.

Under strictly anaerobic conditions, independent of the concentration of yeast present, only 77·4 p.c. of the glucose consumed was fermented to CO_2 and alcohol; the ratio CO_2: alcohol was found to be 1: 1·03 in an average of 16 experiments (theoretical ratio 1: 1·04). 22·6 p.c. of the glucose consumed had been used up in other processes. 65·7 p.c. of the consumed but not fermented glucose could be recovered as reducing substance after hydrolyzing the yeast cells (total carbohydrates). 60·4 p.c. of the non-fermented glucose had been transformed into polysaccharides $(C_6H_{10}O_5)_n$, mainly glycogen and gum besides smaller amounts of membrane polyose (insoluble carbohydrate). The difference between the increase of total carbohydrates and of polysaccharides may be due partly to the production of trehalose (cf. Steiner and Cori, 1935;

Myrbäck, 1936), partly to the presence of traces of hexose phosphoric esters (cf. Macfarlane, 1937; Kruyk and Klingmüller, 1939). Again the separate determinations of the different polysaccharide fractions, especially in low concentrations, involve more losses than the simple hydrolysis by acids (Willstaetter and Rohdewald, 1937).

TABLE 1.

Balance of the fermentation of glucose by compressed yeast (baker's and spirit yeast) under anaerobic conditions.

230·0–750·0 mg. compressed yeast suspended in M/15 KH_2PO_4 solution, 147·2–151·6 mg. glucose purissimum added. Total volume: 15·0 c.c.

pH of the suspension at the beginning of the experiment: 4·3. Gas-space: nitrogen (free of oxygen). Fermented to the complete consumption of the glucose added.[1]

No.	Yeast.[2]	Temperature.	Yeast added, dry weight.	CO_2 produced (a).	Alcohol produced. Experiment (b).	Alcohol produced. Calculated[3] (c).	Increase in dry weight (d).	Correction[4] for the loss of water due to the formation of $(C_6H_{10}O_5)_n$ from $C_6H_{12}O_6$ (e).	Sum of a + b + d + e.	Sum of a + c + d + e.	Glucose added (f).
		°C.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	E	22·5	66·2	57·4	58·8	60·0	29·9	2·1	148·2	149·4	148·6
2	H	22·5	74·3	56·3	60·4	58·8	30·5	2·3	149·5	147·9	147·7
3	H	23	76·5	56·8	59·2	59·4	30·0	2·3	148·3	148·5	149·4
4	H	23	77·4	57·1	57·5	59·7	29·1	2·3	146·0	148·2	148·3
5	E	21	130·6	56·7	58·6	59·3	31·4	2·1	148·8	149·5	150·0
6	E	23	131·4	56·4	59·9	59·0	29·3	2·1	147·7	146·8	148·1
7	E	25	136·9	56·6	57·7	59·1	32·0	2·1	148·4	149·8	149·8
8	E	20	145·5	55·7	56·1	58·2	32·1	2·1	146·0	148·1	147·2
9	H	23	150·3	55·2	57·0	57·7	33·7	2·3	148·2	148·9	148·4
10	H	23	150·7	57·8	59·1	60·3	31·6	2·3	150·8	152·0	151·6
11	H	23	152·4	55·6	56·0	58·1	34·1	2·3	148·0	150·1	150·4
12	H	25	157·8	54·9	57·8	57·4	33·8	2·3	148·8	148·4	147·5
13	H	25	162·7	57·2	58·9	59·8	30·6	2·3	149·0	149·9	150·1
14	E	22	197·2	55·9	57·9	58·4	32·3	2·1	148·2	148·7	149·3
15	Sp	25	219·3	56·0	57·7	58·5	30·7	2·3	146·7	147·5	148·1
16	H	23	246·0	57·1	57·5	59·7	31·2	2·3	148·1	150·3	150·6

[1] A determination by the Hagedorn-Jensen method on the fermented liquid after the experiment showed no detectable glucose in any of the experiments recorded in this table.

[2] E = baker's yeast Effront. H = baker's yeast Hudson. Sp = spirit yeast Hudson.

[3] From (a) according to the equation mg. alcohol = mg. $CO_2 \times \frac{46}{44}$. The agreement between the quantities of alcohol determined and those calculated from the quantity of carbon dioxide produced are within the inevitable errors of the method.

[4] These corrections have been obtained from the average values of i (Table 2) for Hudson and Effront yeast.

One-third of the sugar which was consumed but not fermented is not built up to higher carbohydrates; it must be accounted for as glycerol, traces of acetic acid and a small amount of yeast growth, Wind (1926) having shown that even in 2×10^{-4} vol. p.c. oxygen the growth of yeast is not completely inhibited. In view of the above results an older finding of Harden and Young (1904) is remarkable. These authors found in fermentation experiments with yeast-juice obtained from brewery yeast (the oxygen consumption of this preparation is very small) that on an average 38 p.c. of the sugar disappeared without being accounted for as alcohol or carbon dioxide; it was converted into some compound of less reducing power reconvertible into sugar by hydrolysis with acids. Obviously the zymase system of the yeast-juice contains not only the sugar fermenting enzymes of the living cells but also the glycogen forming ones, a fact nowadays well understood; for it becomes ever more probable (Cori and Cori, 1936–40; Kiessling, 1939; Schaeffner and Specht, 1939) that the formation of glycogen is not an independent process, but rather one connected with the phosphorylating reactions which apparently prepare the 6 carbon atom chain for disruption into two 3 carbon atom compounds.

TABLE 2.

Showing the increase in the total carbohydrates and in the different polysaccharide fractions of the yeast cells after anaerobic fermentation.

No. of experiment shown in Table 1.	Total carbohydrates after hydrolyzing the yeast cells by N. HCl for 4 hr. at 100° C.		Increase in total carbohydrates after fermentation i = h − g.	Glycogen-gum fraction of the yeast cells.		Membrane polyose fraction (insoluble carbohydrate) of the yeast cells.		Increase in polysaccharides after fermentation. o = (l − k) + (n − m)
	Control (g)	After fermentation (h)		Control (k)	After fermentation (l)	Control (m)	After fermentation (n)	
	mg. glucose.	mg. glucose.	mg. glucose.	mg. glucose.	mg. glucose.	mg. glucose.	mg. glucose.	mg. glucose.
1	19·7	41·2	21·5	—	—	—	—	—
2	28·1	52·6	24·5	—	—	—	—	—
3	28·6	48·3	19·7	—	—	—	—	—
4	28·5	50·1	21·6	—	—	—	—	—
5	39·5	60·4	20·9	—	—	—	—	—
6	42·8	61·6	18·8	—	—	—	—	—
7	54·1	79·5	25·4	—	—	—	—	—
8	61·2	82·6	21·4	—	—	—	—	—
9	—	—	—	18·8	34·7	3·6	4·5	16·8
10	—	—	—	26·8	43·9	4·7	7·9	20·3
11	—	—	—	25·6	43·4	5·0	10·1	22·9
12	—	—	—	26·1	45·0	5·8	10·0	23·1
13	—	—	—	25·9	42·3	6·2	11·4	21·6

TABLE 3.

Summarizing the experimental results shown in Tables 1 and 2.

No.	Ratio I. $\frac{\text{Glucose fermented} \times 100}{\text{glucose consumed}}$ $\frac{(a+b)\times 100}{f}$ (alcohol determined).	$\frac{(a+c)\times 100}{f}$ (alcohol calculated from CO_2).	Ratio II. $\frac{\text{Increase in total carbohydrates after fermentation} \times 100}{\text{glucose consumed but not fermented}}$ $\frac{i\times 100}{f-(a+c)}$ (alcohol calculated from CO_2)	Ratio III. $\frac{\text{Increase in polysaccharides after fermentation} \times 100}{\text{glucose consumed but not fermented}}$ $\frac{o\times 100}{f-(a+c)}$ (alcohol calculated from CO_2)
1	78·2	79·0	68·9	—
2	79·0	77·9	75·2	—
3	77·6	77·8	59·3	—
4	77·3	78·8	68·6	—
5	76·9	77·3	61·5	—
6	78·5	77·9	—	51·4
7	76·3	77·2	55·1	—
8	76·0	77·3	—	—
9	75·6	76·1	—	57·2
10	77·1	77·9	75·8	—
11	74·2	75·6	—	62·4
12	76·4	76·1	—	65·6
13	77·3	77·9	—	65·3
14	76·2	76·6	61·1	—
15	76·8	77·3	—	—
16	76·1	77·6	—	—
Average	76·8	77·4	65·7	60·4

TABLE 4.

Demonstrating the influence of oxygen upon fermentation of glucose and formation of higher carbohydrates in comparison with anaerobic conditions.

A. Fermentation in nitrogen, fermentation and respiration in pure oxygen and air.

200·0 mg.[1] fresh compressed yeast suspended in M/15 KH_2PO_4 solution, 150·0–200·0 mg. glucose purissimum added. Total volume: 15·0 c.c. Temperature: 25° C. Time: 2 hours.

Gas-space: nitrogen (free of oxygen) or pure oxygen or air.
Range of O_2-tension in mm. Hg (exclusive of water vapour) from 725 to 600 in pure oxygen.
Range of O_2-tension in mm. Hg (exclusive of water vapour) from 154 to 80 in air.

No.	Yeast.		Dry weight of the yeast. mg.	Fermentation-CO_2 ccm.	Fermentation-CO_2 mg.	Alcohol production. Experiment. mg.	Alcohol production. Calculated. mg.	O_2-uptake. ccm.	$Q_{CO_2}^{N_2}$ (see text).	$Q_{CO_2}^{O_2}$ (see text).	Q_{O_2} (see text).	$\frac{Q_{CO_2}^{N_2} - Q_{CO_2}^{O_2}}{Q_{O_2}}$ (see text).
1	E	in N_2	48·75[2]	17·89	35·15	37·8	36·75	0	183·5	—	—	—
		in air	48·75[2]	7·38	14·5	13·9	15·2	5·40	—	75·7	55·4	1·95
2	H	in N_2	59·4	19·49	38·29	38·7	40·1	0	164·0	—	—	—
		in O_2	58·4	3·76	7·39	6·8	7·7	7·03	—	32·2	60·2	2·19
3	Sp.	in N_2	59·5	20·6	40·47	41·0	42·3	0	173·1	—	—	—
		in O_2 O_2-tension 725–600 mm. Hg	58·63	7·03	13·81	13·7	14·4	7·15	—	60·0	61·0	1·86
		in air O_2-tension 154–92 mm. Hg	58·63	6·76	13·28	15·1	13·9	7·30	—	57·7	62·3	1·85
4	H.	in N_2	31·8	11·72	—	—	—	0	184·3	—	—	—
		in O_2 O_2-tension 725–652 mm. Hg	32·4	2·66	—	—	—	3·82	—	41·1	59·0	2·43
		in air O_2-tension 154–80 mm. Hg	33·5	2·89	—	—	—	4·03	—	43·1	60·0	2·35

[1] In experiment No. 4 125 mg. fresh yeast were used. [2] Performed with the same yeast suspension. [3] Performed with the same yeast suspension.

B. Carbohydrate balance in the presence and in the absence of oxygen, showing that as much higher carbohydrates are synthetized in the absence as in the presence of oxygen.

No. corresp. to Table 4A.		Glucose consumed. (a) mg.	Glucose fermented. (b) mg.	Glucose oxidized. (c) mg.	a − (b + c) mg.	Total carbohydrates after hydrolyzing the yeast cells by N. HCl for 4 hr. at 100° C. Control. (d) mg.	After fermentation. (e) mg.	Increase of total carbohydrates after fermentation. e − d mg.
1	in N_2	96·2	71·9	0	24·3	16·3	30·2	13·9
	in air	67·6	29·7	7·2	30·7	16·3	30·6	14·3
2	in N_2	101·4	78·4	0	23·0	21·0	33·5	12·5
	in O_2	56·8	15·1	9·4	32·3	20·1	34·9	14·8
3	in N_2	108·2	82·8	0	25·4	19·4	32·6	13·2
	in O_2	70·3	28·2	9·6	32·5	18·5	33·2	14·7
	in air	68·0	27·2	9·8	31·0	18·5	33·9	15·4

Comparison of the Carbohydrate Balance Under Aerobic and Under Anaerobic Conditions.

In the Tables 4A and 4B are recorded the results of experiments which demonstrate the influence of oxygen on the alcoholic fermentation of glucose and the formation of higher carbohydrates; corresponding experiments under strictly anaerobic conditions were performed to permit a comparison. The experiments of this section were carried out with 200 mg. fresh yeast (experiment No. 4 with 125 mg. fresh yeast) and an excess of glucose so that the final concentration at the end of the experiment did not drop below 0·5 p.c. In the presence of oxygen (or air) the total amount of glucose used up is diminished by about 30–45 p.c. compared with the consumption of glucose under anaerobic conditions. The rate of fermentation decreases by about 60–80 p.c. Part of the glucose is oxidized and the Meyerhof quotient $\frac{Q_{CO_2}^{N_2} - Q_{CO_2}^{O_2}}{Q_{O_2}}$, i.e. the ratio between inhibited fermentation and respiration, varies between 1·8 and 2·4. There remains a considerable amount of the glucose—much more than under anaerobic conditions—which has not been used up either by fermentation or by respiration (Table 4B). Although in the presence of oxygen (or air) the amount of glucose consumed but not broken down is larger than in nitrogen, the amount of higher carbohydrates formed from this glucose is of the same order of magnitude as under anaerobic conditions. No support could be obtained for the presence of difficultly hydrolyzable carbohydrates, as suggested by Lundin (1923). *The yeast does not form more higher carbohydrates in the presence than it does in the absence of oxygen.* Besides the production of higher carbohydrates under aerobic conditions a synthesis of fat (P. Lindner, 1919; Smedley-Maclean, 1922) and a somewhat larger protein synthesis (yeast growth) may occur together with the formation of subsidiary products.

The examples Nos. 3 and 4 in Table 4A show that the respiration and the Pasteur effect of compressed yeast are independent of oxygen tension within the range of O_2 tension between 725 and 80 mg. Hg (exclusive of water vapour) at 25° C. It has been proved for fresh yeast (Cook, 1930) and many other cells, e.g. bacteria, eggs of sea-urchin, red blood corpuscles (Warburg, 1914), that the rate of O_2 consumption is unaffected by oxygen tensions above definite critical values. Below the critical value the rate of O_2 uptake varies rapidly with O_2 tension yielding a hyperbolic relation. The value of the critical tension changes with temperature (Shibata and Tamiya, 1930; Stier, 1935). Cook (1930) found for yeast at 37° C. 100 p.c. respiration at 144 mm. Hg O_2 tension (total pressure maintained at 760 mm. Hg), 97 p.c. respiration at 64 mm. tension, 84 p.c. respiration at 36 mm. and 60 p.c. respiration at 11·5 mm. Keeping above the critical oxygen tension I never observed that decrease of oxygen tension (725 → 80 mm. Hg) caused any increase in aerobic fermentation outside the error of the method. Leiner (1935) (in Warburg's laboratory) using nucleated red blood corpuscles as respiratory subjects demonstrated that variations of the oxygen tension from 676 to 43 mm. Hg did not effect the rate of aerobic glycolysis. In view of these results the opinion seems to be justified that in the presence of oxygen it is the respiration which suppresses fermentation and that this effect is independent of the oxygen tension above the critical value. The assumption suggests itself that the contradictory findings of Bumm *et al.* (1934) and Laser (1937) working with various animal tissues (mucosa of the intestine, retina, chorion, allantois, liver, tumour) are due to the fact that cells connected to tissues are unsuitable for respiratory experiments at low oxygen tension because of the danger of insufficient O_2 diffusion into the cells; moreover any decrease in O_2-uptake is out of proportion to the increase in aerobic fermentation so that small decreases in respiration cause considerable increases in aerobic fermentation; this phenomenon can be regularly observed in experiments with yeast if the oxygen tension is allowed to drop 10–20 mm. Hg below the critical value for 20–30 minutes and the explanation of this is given in the discussion of the mechanism of the Pasteur effect.

The calculations of Table 4 (A and B) are based on the assumption that the R.Q. equals unity. In biological systems such as yeast in which both respiration and fermentation take place simultaneously a direct determination of the R.Q. by volumetric gas measurements and analysis of the final gas mixture is impossible.

Let a = total CO_2 produced by respiration and fermentation, b = respiration CO_2, c = fermentation CO_2,
therefore $a = b + c$ (experimentally measured) (1)
let d = oxygen uptake (experimentally measured) (2)
let e = change in total volume (final volume—initial volume)
therefore $e = a - d$ (experimentally measured) (3)

$$\text{now} \quad \text{R.Q.} = \frac{b}{d} = \frac{a - c}{d} = \frac{a - c}{a - e}, \tag{4}$$

in this equation only a and d, a and e respectively are known. To determine the R.Q. either b or c must be determined by another independent method. Such a method is the determination of the alcohol produced in aerobic fermentation. The production of 1 mol ethyl alcohol being equivalent to the production of 1 mol fermentation CO_2, the alcohol determination fixes the value of c in equation 4. Calculating the R.Q. of the experiments in Table 4A on that basis the following results are obtained: 1·11; 1·06; 0·93; 1·04.

Balance of the Fermentation of Fructose.

The fact that yeast does not produce more higher carbohydrates in the presence than in the absence of oxygen is not compatible with the existence of a Meyerhof cycle which would demand the synthesis of more carbohydrate under aerobic conditions as a result of the oxidative resynthesis. There remains still the possibility, referred to by Meyerhof and Finkle (1925) for the carbohydrate cycle in lactic acid bacteria, that in the presence of oxygen the yeast synthetizes glucose from intermediates within the cell and secretes the sugar into the surrounding fluid. To test this possibility glucose was replaced by fructose and after two hours of fermentation simultaneous determinations of the reducing power and the rotatory power of the residual sugar were made (Table 5).

TABLE 5.

Balance of the fructose fermentation under anaerobic and aerobic conditions.

200·0 mg. fresh compressed spirit yeast (Hudson) suspended in M/15 KH_2PO_4 solution. 135–201 mg. fructose added. Total volume: 15·0 c.c. Temperature: 25° C.

Gas space: nitrogen (free from oxygen) or pure oxygen or air.

No.		Time, minutes.	Dry weight of the yeast. mg.	Fructose added. mg.	Fermentation CO_2. c.c.	O_2 consumed. c.c.	$Q_{CO_2}^{N_2}$	$Q_{CO_2}^{O_2}$	Q_{O_2}	$\frac{Q_{CO_2}^{N_2} - Q_{CO_2}^{O_2}}{Q_{O_2}}$	Sugar left after fermentation. Reducing substances (Hagedorn-Jensen). mg. in 15 c.c.	Polarimeter reading (A) in 1d-tube 5461 A°U.	Amount of glucose and fructose calculated from equation I and II.[1] Fructose. mg. in 15 c.c.m.	Glucose. mg. in 15 c.c.m.
											100 of the original fructose contained		97·7	2·3
1	in N_2	105	52·7	200·9	18·84	0	204·3	—	—	—	99·0	— 0·67°	94·9	4·1
	in air	105	53·6	135·0	7·58	6·52	—	80·8	69·5	1·78	65·7	— 0·43°	61·7	4·0
2	in air	95	53·4	142·8	8·28	5·26	—	97·9	62·2	—	70·5	— 0·47°	66·9	3·6
3	in O_2	110	58·6	160·4	10·53	5·72	—	98·0	53·2	—	78·8	— 0·54°	76·1	2·7
4	in O_2	105	57·2	179·5	9·42	5·61	—	94·1	56·0	—	101·7	— 0·69°	97·6	4·1

[1] Equation I: $\chi = \frac{100A - [\alpha_2]_{Hg}^{20°} \cdot P}{[\alpha_1]_{Hg}^{20°} - [\alpha_2]_{Hg}^{20°}}$; equation II: $y = \frac{[\alpha_1]_{Hg}^{20°} \cdot P - 100A}{[\alpha_1]_{Hg}^{20°} - [\alpha_2]_{Hg}^{20°}}$.

χ = gm. fructose per 100 mls. solution.
y = gm. glucose per 100 mls. solution.
P = gm. reducing substances per 100 mls. solution.
A = polarimeter reading in 1d-tube; 5461 A.°U; at 20° C.
$[\alpha_1]_{Hg}^{20°}$ = specific rotation of d-fructose.
$[\alpha_2]_{Hg}^{20°}$ = specific rotation of d-glucose.

With fructose as substrate a Pasteur effect similar to that with glucose is observed. The sugar, however, not consumed by the yeast in the presence of oxygen is the laevo-rotatory fructose; no appreciable glucose formation occurs. The suggestion that in the case of aerobic glucose fermentation glucose is resynthetized, while in the case of aerobic fructose fermentation fructose is built up seems to be not acceptable. The theory of Meyerhof (1925) that the Pasteur effect in alcoholic fermentation by yeast cells is due to an oxidative resynthesis must accordingly be rejected.

Fermentation of Acetaldehyde Under Anaerobic and Aerobic Conditions.

If the mechanism of the Pasteur effect cannot be explained by the existence of a Meyerhof cycle we are left with the other possibility that the gross rate of carbohydrate breakdown is directly inhibited by the presence of oxygen (cf. A. Hahn, 1931; Lipmann, 1933). Information on the mechanism through which the respiration causes the inhibition of alcoholic fermentation was provided by experiments concerning the fate of acetaldehyde in contact with living yeast under anaerobic and aerobic conditions.

Anaerobic conditions. When yeast acts upon acetaldehyde under anaerobic conditions part of the acetaldehyde disappears yielding equimolecular amounts of ethyl alcohol and acetic acid. In principle this fact has been known since Kostytschew (1914) and Neuberg (1914). However, since the experiments of these authors were performed neither under strictly anaerobic conditions nor with agitation of the yeast suspension, no exact figures of the acetaldehyde consumption per unit weight and time are obtainable from these early experiments.

TABLE 6.

Acetaldehyde dismutation under anaerobic conditions.

Authors.	Experimental conditions.	Time, minutes.	Acetaldehyde consumed. mg.	Alcohol produced. mg.	Acetic acid produced. mg.	Acetaldehyde consumed per 100 mg. yeast dry weight and hour. anaerobically.	Acetaldehyde consumed for 100 mg. yeast dry weight and hour. aerobically.[1]
Wieland and Claren	Impoverished brewery yeast (bottom yeast) 265 mg. dry weight, M/30 acetaldehyde. total volume: 27 c.c. 30°C.; shaken	150	23·8	10·6	18·6	3·6	4·4
		150	19·8	10·1	18·6	3·0	4·8
Heicken	Impoverished brewery yeast 348 mg. dry weight, M/36 acetaldehyde. Total volume: 53 c.c. 30°C.; shaken	30	9·2	—	—	5·3	7·8
		60	18·9	—	—	5·4	9·0
		120	34·8	—	—	5·0	8·2
		180	45·8	—	—	4·4	6·7
							11·9
Gottschalk	Compressed yeast (baker's yeast) 52 mg. dry weight, M/50[2] acetaldehyde. Total volume: 15 c.c. 25°C.; shaken						11·7
		120	2·5	1·1	1·5	2·4	10·8
		120	2·8	1·2	1·5	2·7	11·5
							12·1
							11·6
							12·0

[1] The aerobic experiments were performed under corresponding conditions, but not with the same yeast-suspension.

[2] Anaerobic experiments with M/40 acetaldehyde had the same result.

In Table 6 the figures of Wieland and Claren (1932) and of Heicken (1938), both authors having used "impoverished" brewery yeast (Munich bottom yeast), are compared with those which I have obtained in anaerobic experiments with compressed yeast. The right to consider the volatile acid as acetic acid as is done in the experiments of Table 6 results from the investigations of Kostytschew and Neuberg in which the acetic acid was identified by its silver salt and in which it was shown that the acetic acid is practically the only volatile acid formed by yeast in contact with acetaldehyde. In control experiments it was found that 200 mg. fresh compressed yeast do not contain measurable amounts of alcohol or acetaldehyde; the content of volatile acids, calculated as acetic acid, was below 0·15 mg. The balance of the acetaldehyde metabolism under anaerobic conditions is:

$$CH_3.CHO + CH_3.CHO + H_2O \longrightarrow CH_3.CH_2OH + CH_3.COOH \qquad (5)$$

Aerobic conditions. In the presence of oxygen compressed yeast consumes 360 p.c. more acetaldehyde than anaerobically, while with brewery yeast the increase amounts only to 40-58 p.c. This is due to the fact that the oxidative system in the compressed yeast (baker's and spirit yeast) is much more developed than in the brewery yeast (Meyerhof, 1925; Euler *et al.*, 1939; Fink and Lechner, 1940) which Wieland and Claren (1932) and Heicken (1938) used for investigations on the oxidation of acetaldehyde.

TABLE 7.

Acetaldehyde metabolism by compressed yeast under aerobic conditions. Experiments with sufficient supply of acetaldehyde.

200 mg. fresh compressed yeast (baker's yeast Effront) suspended in M/15 KH_2PO_4 solution. 0·48–0·70 c.c. 2·8 p.c. acetaldehyde solution added. Total volume: 15·0 c.c. Temperature: 25° C. Time: 2 hours. Gas-space: air.

	Number of experiment.	I	II	III	IV	V	VI	VII
a	Dry weight of the yeast, mg.	54·4	53·3	54·9	55·2	52·0	56·8	53·6
b	Acetaldehyde added, mg.	19·6	16·8	16·2	14·0	14·2	13·2	13·0
c	Acetaldehyde recovered, mg.	6·8	4·4	4·3	1·3	1·6	0	0
d	CO_2 produced, c.c.	5·20	5·36	5·13	5·59	4·99	5·54	5·63
e	O_2 consumed, c.c.	5·01	5·44	5·22	5·50	4·95	5·69	5·71
f	Respiratory quotient	1·04	0·99	0·98	0·98	1·01	0·97	0·99
g	Alcohol formed, mg.	6·1	6·17	5·8	6·2	6·6	6·55	6·5
h	Acetic acid formed, mg.	1·2	1·0	1·0	0·4	not directly estimated		
i	Q_{O_2} calculated from a and e	46·1	51·0	47·5	49·8	47·6	50·1	53·3
k	Q_{O_2} in control experiment with glucose as substrate	59·5	62·3	61·4	64·6	—	—	—
l	Acetaldehyde oxidized to CO_2 and H_2O, calculated from d, mg.	5·11	5·26	5·04	5·49	4·90	5·44	5·53
m	Acetaldehyde reduced to ethyl alcohol, calculated from g, mg.	5·83	5·90	5·55	5·93	6·31	6·26	6·22
n	Acetaldehyde transformed into acetic acid, calculated from h, mg.	0·9	0·7	0·7	0·3	1·41	0·82	0·69
						calculated by m–l		
o	*Acetaldehyde consumption.*							
	Calculated from the reaction products, mg.	11·84	11·86	11·29	11·72	12·62	12·52	12·44
	Calculated from initial and final concentrations (b–c)	12·8	12·4	11·9	12·7	12·6	13·2	13·0

Furthermore I observed (Table 7) that 43 p.c. of the acetaldehyde consumed is completely oxidized to CO_2 and H_2O, 7 p.c. transformed into acetic acid and 50 p.c. reduced to ethyl alcohol. The respiratory quotient is 1·0. These results could be reproduced very regularly provided that the acetaldehyde supply was sufficient (at least 13 mg. acetaldehyde per 50 mg. yeast dry weight, 2 hr. at 25° C). I conclude from these experimental data that coupled with one step in the

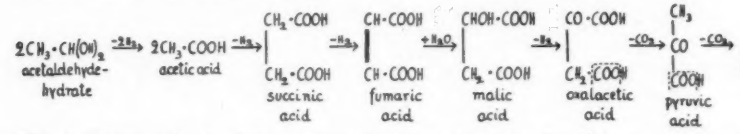
oxidation of acetaldehyde to CO_2 and H_2O is the reduction of an equivalent amount of acetaldehyde to ethyl alcohol according to the equation:

$$CH_3.CHO + 2O_2 \longrightarrow 2CO_2 + 2H_2O \qquad \text{R.Q.} = 1{\cdot}0 \left(\frac{2CO_2}{2O_2}\right) \qquad (6)$$

$$CH_3.CHO + \overset{O}{\overset{\|}{H_2}} \longrightarrow CH_3.CH_2OH$$

Equation 6 explains the origin of most of the alcohol produced and the value for the respiratory quotient. The alcohol formed above the amount equivalent to the acetaldehyde oxidation can only come from a dismutation according to equation 5 which also proceeds—with decreased velocity—in the presence of oxygen and which produces the equimolecular amount of acetic acid, found in experiments I–IV (Table 7).

The conclusion drawn by Heicken from his experimental figures (cf. original) that the total amount of acetaldehyde consumed is at first dismutated to ethyl alcohol and acetic acid and the latter is then selectively oxidized to CO_2 and H_2O is not tenable. Did this occur the velocity of the dismutation of the acetaldehyde would be the limiting factor for the velocity of the acetaldehyde oxidation. From Table 6 however it can be seen that with compressed yeast as well as with brewery yeast the velocity of the acetaldehyde metabolism is greater under aerobic than under anaerobic conditions. Wieland and Claren (1932) whose experimental results differ from those of Heicken (1938) suppose in view of the higher rate of acetaldehyde consumption in the presence than in the absence of oxygen that under aerobic conditions only part of the acetaldehyde is dismutated to alcohol and acetic acid, another part being dehydrogenated directly to acetic acid which is then submitted to the Thunberg cycle (Wieland and Sonderhoff, 1932):



The authors admit that an explanation for the fact that under aerobic conditions more ethyl alcohol is produced than under anaerobic conditions cannot be given. Against the theory commonly accepted that the first step of the direct acetaldehyde oxidation in yeast cells is the reaction $CH_3.CH(OH)_2 \xrightarrow{-H_2} CH_3.COOH$ two objections may be made:

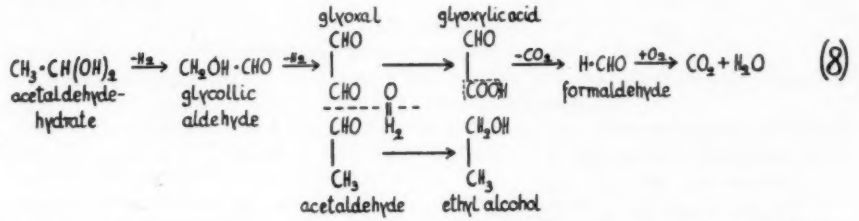
1. If acetic acid is the first intermediate product in the oxidation of acetaldehyde to CO_2 and H_2O it would be expected that in a mixture of acetic acid and ethyl alcohol the former would be selectively oxidized before the latter was attacked; for ethyl alcohol is always present in aerobic acetaldehyde fermentation and is not attacked so long as acetaldehyde is present as substrate (Table 7). Contrary to the theory Heicken (1938) has observed that yeast, suspended in a mixture of ethyl alcohol and acetic acid in adequate amounts, oxidizes selectively the alcohol before attacking the acetic acid. This agrees with the chemical experience that *in vitro* the ethyl alcohol is oxidized more readily than acetic acid. The argument that there is a difference between the acetic acid added and the active acetic acid produced upon the active surface of the acetaldehyde dehydrogenase is not attractive in this case. The findings of Wieland and Claren (1932), Heicken (1938) and myself agree in the point that that part of the acetic acid which comes from the dismutation of part of the acetaldehyde consumed and which also must be regarded as acetic acid *in statu nascendi* is not attacked by the yeast enzymes so long as acetaldehyde or alcohol is present.

2. These experiments prove that besides the dismutation of a small amount of acetaldehyde according to equation 5 the oxidation of one molecule of acetaldehyde to CO_2 and H_2O is coupled with the reduction of another molecule of acetaldehyde to ethyl alcohol. It would be very difficult to fit in a coupled oxidation-reduction in the series of those reactions which according to Wieland and Sonderhoff (1932) take place in the enzymatic acetaldehyde oxidation (equation 7).

It seems, therefore, that the theory discussed can hardly be reconciled with the experimental findings.

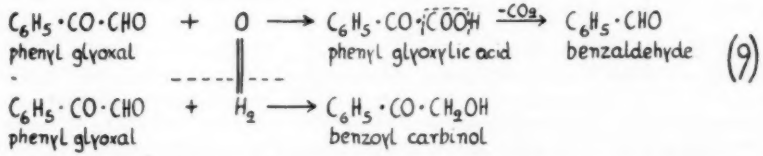
I suggest that in yeast cells the first reaction product of the enzymatic acetaldehyde oxidation is not acetic acid but rather glycollic-aldehyde and that the

glycollic-aldehyde, a very labile and reactive substance, is further oxidized in the following manner:



(8)

An alternative scheme is to dismutate glycollic-aldehyde and acetaldehyde yielding glycollic acid and ethyl alcohol. The evidence of Wieland and Sonderhoff (1932) renders this scheme the less probable. The reduction of one molecule of acetaldehyde to ethyl alcohol fits as a coupled reaction very well into this series of reactions which would be initiated by an *α-oxidation of the acetaldehyde* activated by an *acetaldehyde α-dehydrogenase*. The mixed dismutation between acetaldehyde and glyoxal would be the counterpart of the mixed dismutation between acetaldehyde and 1:3-diphosphoglyceraldehyde which is the essential reaction of the alcoholic fermentation. The readiness of the glyoxal for dismutation and the glyoxylic acid for decarboxylation has been proved by Dakin (1914). Adding phenyl glyoxal to fermenting yeast Dakin isolated benzoyl carbinol, phenyl glyoxylic acid and benzaldehyde besides l-mandelic acid and benzyl alcohol as reaction products. It is very probable that besides other reactions the following dismutation and decarboxylation take place:



(9)

The theory of the α-oxidation of the acetaldehyde by an α-dehydrogenase, though not opposed to any fact known, demands further experimental foundation. Mr. H. F. Holden and I have tackled this problem and we shall report jointly the results in a later paper.

In the experiments described above there was either an excess of acetaldehyde present or just sufficient to satisfy the requirements of the oxidative system of the yeast. If, however, the acetaldehyde supply is insufficient the yeast starts to oxidize the alcohol which in the presence of acetaldehyde down to very low concentrations is practically not attacked. The oxidation of this alcohol causes a decrease in the respiratory quotient, in my experiments down to 0·8. From the results of Wieland and Wille (1933) and Heicken (1938) it is known that the R.Q. of alcohol oxidation within the first two hours is about 0·5.

In Table 8 the experimental figures are calculated on the basis that 43 p.c. of the acetaldehyde consumed has been oxidized to CO_2 and H_2O (cf. Table 7), 43 p.c. reduced to ethyl alcohol and the rest dismutated to acetic acid and alcohol. The difference between the alcohol formed in these reactions and the amount of alcohol experimentally recovered is supposed to have been oxidized with an R.Q. = 0·5. Table 8 shows that the figures for O_2 consumption and CO_2 production calculated on this basis are in close agreement with the experimental figures for O_2 uptake and CO_2 production. In experiments with M/100–M/200 acetaldehyde concentrations Meyerhof (1925) ascribed the low R.Q. observed to the fact that per molecule of CH_3.CHO totally oxidized one molecule is synthetized to carbohydrate. The low R.Q. (0·7) in Meyerhof's (1925) experiments is due to a secondary alcohol oxidation because of very low acetaldehyde concentration. A carbohydrate synthesis from acetaldehyde or some oxidative intermediate does not take place. I have a few times controlled the acetaldehyde balances by determinations of the dry weight before and after aerobic acetaldehyde fermentation, but have never found any increase in

dry weight. For instance in experiment No. V (Table 7) the dry weight of 5 c.c. of the yeast suspension at the beginning of the experiment was 62·5 mg., the corresponding dry weight at the end of the experiment 62·1 mg.

TABLE 8.

Experiments with insufficient supply of acetaldyhyde.

	Measurements.	Number of experiment. I	II	III
a	Dry weight of the yeast, mg.	54·2	53·5	56·7
b	Acetaldehyde added, mg.	12·2	11·0	9·5
c	Acetaldehyde recovered, mg.	0	0	0
d	CO_2 produced in c.c.	5·64	5·0	4·93
e	O_2 consumed in c.c.	6·02	6·25	6·04
f	Respiratory quotient	0·94	0·80	0·81
g	Alcohol found, mg.	5·3	3·7	2·8
	Calculations from above results based on the assumptions mentioned in the text.			
h	Acetaldehyde oxidized, mg.	5·25	4·73	4·08
	O_2 consumption in c.c. (respiratory quotient = 1·0)	5·35	4·82	4·15
	CO_2 production in c.c.	5·34	4·81	4·15
i	Alcohol production coupled with h, mg.	5·49	4·94	4·27
	Alcohol production by dismutation, mg.	0·89	0·80	0·70
k	Mg. alcohol oxidized (i–g) with respiratory quotient = 0·5	1·08	2·04	2·17
	O_2 consumption in c.c.	1·05	1·99	2·11
	CO_2 production in c.c.	0·52	0·99	1·06
l	Total O_2 consumption calculated, c.c.	6·40	6·81	6·26
	Total O_2 consumption experimentally, c.c.	6·02	6·25	6·04
m	Total CO_2 production calculated, c.c.	5·86	5·80	5·21
	Total CO_2 production experimentally, c.c.	5·64	5·0	4·93

The experiments demonstrate that yeast cells in contact with a mixture of acetaldehyde, ethyl alcohol and acetic acid attack at first selectively the aldehyde. Down to very low concentrations the acetaldehyde remains the only substrate for the respiratory system of the yeast.

A comparison of the amounts of glucose and of acetaldehyde oxidized by the same amount of oxygen shows the following:

$$1\ \text{mol}\ C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O \qquad (10)$$

or

$$1\ \text{mol.}\ CH_3.CHO + 2\tfrac{1}{2}O_2 \longrightarrow 2CO_2 + 2H_2O$$

$$2{\cdot}4\ \text{mol}\ CH_3.CHO + 6O_2 \longrightarrow 4{\cdot}8CO_2 + 4{\cdot}8H_2O \qquad (11)$$

When the same amount of oxygen is taken up in the experiments with glucose and acetaldehyde as substrates, the oxidation of 1 molecule of glucose is equivalent to the oxidation of 2·4 molecules of acetaldehyde. In the experiments of Table 7 on an average 9·0 mg. glucose and 5·2 mg. acetaldehyde were oxidized, i.e. the ratio mol glucose : mol acetaldehyde is 1 : 2·36. The oxygen consumption in the experiments with acetaldehyde as substrate was in fact only 80 p.c. of the value with glucose as substrate (cf. Table 7 k). That is due to the fact that in the case of acetaldehyde oxidation 20 p.c. of the oxygen required is provided by a coupled reduction. From these experiments it can be concluded that the acetaldehyde fulfils the requirement of a substance supposed to be an intermediate in the respiratory process of an organism: its velocity of reaction with oxygen is of the same order as that of the oxidative reactions of the complete respiratory system.

THE MECHANISM OF THE PASTEUR EFFECT.

After the cleavage of the 6-carbon chain the mixed dismutaton between 1 molecule of triosephosphate and 1 molecule of acetaldehyde occurs in alcoholic

fermentation under anaerobic conditions. Negelein and Wulff (1937) and Warburg (1939) have shown that this dismutation is activated by two different enzymes with diphosphopyridine nucleotide (= cozymase = coenzyme I = CoI) as hydrogen carrier; the reacting triosephosphate is the phosphorylated Fischer ester (Negelein and Brömel, 1939):

$$\begin{array}{l}\text{1: 3-diphosphoglyceraldehyde} + H_2O + \text{CoI} \rightarrow \text{CoI}H_2 + \text{1: 3-diphosphoglyceric acid} \\ \text{acetaldehyde} + \text{CoI}H_2 \longrightarrow \text{alcohol} + \text{CoI} \qquad (12)\end{array}$$

As the reduction of acetaldehyde to ethyl alcohol in yeast cells is possible only by means of the reduced pyridine nucleotide equations (5) and (6) must be formulated in the following manner:

$$\begin{array}{l}\text{acetaldehyde} + H_2O + \text{CoI} \longrightarrow \text{CoI}H_2 + \text{acetic acid} \\ \text{acetaldehyde} + \text{CoI}H_2 \longrightarrow \text{ethyl alcohol} + \text{CoI} \qquad (13)\end{array}$$

$$\begin{array}{l}\text{acetaldehyde} + 2O_2 + H_2O + \text{CoI} \longrightarrow \text{CoI}H_2 + 2CO_2 + 2H_2O \\ \text{acetaldehyde} + \text{CoI}H_2 \longrightarrow \text{ethyl alcohol} + \text{CoI} \qquad (14)\end{array}$$

In all three cases (12-14) the regeneration of the reduced cozymase ($\text{CoI}H_2$) is effected by acetaldehyde as hydrogen acceptor and a protein catalyst. The yeast contains still another system able to reoxidize $\text{CoI}H_2$. In animal tissues as well as in yeast a dehydrogenase for the reduced coenzyme I, named "diaphorase I", occurs which catalyses the transport of hydrogen from dihydropyridine-nucleotide to acceptors like cytochrome or methylene blue but not to molecular oxygen (Euler *et al.*, 1938; Dewan and Green, 1938). The reduced cytochromes (probably cytochrome $a+b$) are in further steps reoxidized by cytochrome oxidase and molecular oxygen:

$$\text{reduced coenzyme I} \xrightarrow[\text{diaphorase I}]{} \text{cytochrome } a, \text{cytochrome } b \xrightarrow[\text{cytochrome-oxidase}]{} O_2$$

the arrows show the direction of hydrogen transfer.

$$\text{diaphoraseI} + \text{cytochromes} + \text{cytochrome-oxidase} + O_2 = \text{Diaph-Cy-}O_2.$$

$$\text{diaphoraseI} + \text{cytochromes} + \text{cytochrome-oxidase} = \text{diaphorase-cytochrome system.}$$

Under anaerobic conditions $\text{CoI}H_2$ is reoxidized by acetaldehyde. Even in the presence of oxygen the reoxidation of $\text{CoI}H_2$ is affected by acetaldehyde if there is acetaldehyde available. This fact has been proved by the experiments with acetaldehyde as substrate in the presence of oxygen. We must conclude from this finding that in yeast cells the velocity of the reaction between dihydropyridine-nucleotide and acetaldehyde (activated by a specific protein) greatly exceeds the velocity of that reaction in which the reoxidation of $\text{CoI}H_2$ is effected by Diaph–Cy–O_2. Only when there is no acetaldehyde available, $\text{CoI}H_2$ is regenerated by Diaph–Cy–O_2.

TABLE 9.

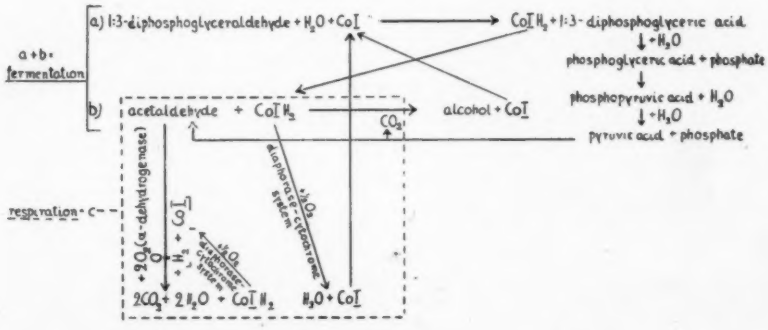


Table 9 illustrates the reactions taking place in aerobic glucose fermentation by yeast cells. As already shown (Gottschalk, 1923) even in the presence of oxygen the pyruvic acid is at first decarboxylated by yeast before further changes take place. The acetaldehyde, thus formed is partly oxidized to CO_2 and H_2O, partly reduced to ethyl alcohol. Only part of the reduced cozymase is reoxidized by acetaldehyde; the remainder has to be regenerated by the Diaph–Cy–O_2 because no more acetaldehyde is available. The conditions in aerobic glucose fermentation differ from those in aerobic acetaldehyde fermentation in one essential point: in aerobic glucose fermentation each molecule of acetaldehyde oxidized is accompanied by one molecule of $CoIH_2$, due to reaction a; a second molecule of $CoIH_2$ is formed in the course of the aldehyde oxidation; both molecules of $CoIH_2$ can only be reoxidized by the Diaph–Cy–O_2 because there is no acetaldehyde available as H_2 acceptor. The reoxidation by means of the Diaph–Cy–O_2, however, is slower than the reoxidation by acetaldehyde as H_2 acceptor. This slower reoxidation of $CoIH_2$ in turn causes the decrease in the total amount of carbohydrate destruction; the diminished velocity of the reoxidation of the reduced cozymase becomes the factor determining the velocity of the carbohydrate metabolism of the yeast under aerobic conditions. *The Pasteur effect is, in the last analysis, due to the circumstance that in the presence of oxygen part of the cozymase present in the yeast cell is put out of action by persisting in the reduced state.* According to Ball (1939), who also emphasized the rôle of the diphosphopyridine nucleotide, "the action of oxygen in reducing the rate of carbohydrate destruction must be sought in the fact that the aerobic process by its complete combustion makes available the total energy of the carbohydrate molecule. The anaerobic process on the other hand by its incomplete combustion liberates only a small part of the available energy of the carbohydrate. Hence to furnish the same amount of energy the rate of carbohydrate disappearance must be greater under anaerobic conditions than when oxygen is present". This explanation cannot be accepted because the decrease in the total amount of carbohydrate destruction in the presence of O_2 is only $\frac{1}{3}$ to $\frac{1}{2}$, while it should be far larger if due to requirements of energy, since 1 molecule of glucose completely oxidized in respiration yields more energy than 25 molecules of glucose fermented to CO_2 and alcohol. Stern (1940), too, has criticized Ball's theory.

The theory of the mechanism of the Pasteur effect, developed here, is in agreement with the facts as yet known about this effect and is able to explain observations until now difficult of interpretation. The complete inhibition of the Pasteur effect by cyanide (Warburg, 1926b) is natural since we know that the acetaldehyde dehydrogenase is cyanide-sensitive (cf. M. Dixon *et al.*, 1937; Gordon, Green and Subrahmanyan, 1940; see also Genevois, 1927); but in addition Warburg (1926b) has found that aerobic fermentation of yeast, supposed to be dependent on the magnitude of respiration, is only partly dependent on respiration in presence of carbon monoxide; it increases with decrease of respiration, but not proportionally; in cases where respiration was inhibited to the extent of 24 p.c. by CO, the decrease in respiration could not account for the 100 p.c. increase in aerobic fermentation; Warburg therefore supposed a selectively inhibitory effect of CO upon the Pasteur reaction disrupting the coupling normally existing between respiration and fermentation. Very similar observations were made with isocyanide (Warburg, 1926a). The partial lack of dependence of the fermentation on respiration in the presence of CO or isocyanide is only apparent. It can be seen from Table 9 that any decrease in reaction c is followed by an increase in reaction b with an accompanying increase in reoxidized $CoIH_2$. Again the decreased amount of reaction c diminishes the amount of reduced cozymase connected therewith to be reoxidized by the diaphorase-cytochrome system. This frees some of the latter. Thus the proportion of the cozymase persisting in the reduced form

is diminished both due to direct reduction of acetaldehyde and to the action of the freed part of the diaphorase-cytochrome system in reoxidizing some of the already persisting reduced cozymase. Therefore the increase in fermentation is disproportionally greater than the decrease in respiration. The effectiveness of the respiration upon the fermentation, i.e. the Meyerhof quotient $\frac{Q_{CO_2}^{N_2} - Q_{CO_2}^{O_2}}{Q_{O_2}}$, depends

1. on the velocity of acetaldehyde oxidation, and
2. on the velocity of the reoxidation of the $CoIH_2$ by Diaph–Cy–O_2.

Variations of the Meyerhof quotient in different yeast races or different samples of the same race under the same experimental conditions (pH, temperature, concentration of substrate, ionic strength, etc.) are apparently due to variations in the concentration of the acetaldehyde dehydrogenating enzyme system or of the diaphorase-cytochrome system or of both.

The mechanism of the Pasteur effect as explained above, has been experimentally verified for the fermentation of sugar by yeast cells. It is possible that a similar sequence may adequately describe the Pasteur effect as observed in animal cells. In animal cells—under anaerobic conditions—the reoxidation of $CoIH_2$ is effected according to the equation: pyruvic acid + $CoIH_2$ ⟶ lactic acid + CoI (15). In the presence of oxygen the pyruvic acid is totally or partly oxidized to CO_2 and H_2O and the $CoIH_2$ thus not regenerated according to equation (15) has to be reoxidized by the Diaph–Cy–O_2.

SUMMARY.

In experiments with fresh compressed yeast under strictly anaerobic conditions it was shown that only 77·4 p.c. of the glucose added is fermented to CO_2 and alcohol. 65·7 p.c. of the glucose consumed but not fermented is reconvertible into reducing substance by acid hydrolysis. 60·4 p.c. of the non-fermented glucose is built up to polysaccharides, mainly glycogen and gum besides smaller amounts of membrane polyose. A comparison of the total carbohydrate balance of alcoholic fermentation in the presence and absence of oxygen showed that compressed yeast does not synthetize more higher carbohydrates under aerobic than under anaerobic conditions though in the presence of O_2 the amount of glucose consumed but not accounted for by fermentation and respiration is larger than in the absence of O_2. When fructose is being aerobically fermented, the residual sugar is fructose; no glucose is formed. These results are incompatible with the existence of a Meyerhof cycle. Furthermore it was observed that the Pasteur effect is independent of oxygen tension between O_2 tensions of 725 to 80 mm. Hg. (exclusive of water vapour).

In the experiments with acetaldehyde as substrate it was established that compressed yeast consumes 360 p.c. more acetaldehyde aerobically than anaerobically; therefore the first step of the oxidative metabolism of acetaldehyde cannot be a dismutation. Moreover it was found that coupled with one step of acetaldehyde oxidation to CO_2 and H_2O is the reduction of an equivalent amount of acetaldehyde to ethyl alcohol. In view of these findings and the fact that acetic acid is not attacked by living yeast as long as acetaldehyde or ethyl alcohol is available as respiratory substrate it is concluded that the first step of the enzymatic acetaldehyde oxidation is an α–oxidation, catalysed by an α–dehydrogenase and yielding glycollic aldehyde which is further oxidized via glyoxal, glyoxylic acid, formaldehyde to CO_2 and H_2O, the oxidation glyoxal $\xrightarrow{+O}$ glyoxylic acid being coupled with the reduction acetaldehyde $\xrightarrow{+H_2}$ ethyl alcohol. The velocity of glucose oxidation and acetaldehyde oxidation by compressed yeast is the same.

Under anaerobic conditions the dihydro-cozymase ($CoIH_2$) is reoxidized by

acetaldehyde as H_2 acceptor; aerobically O_2 regenerates part of $CoIH_2$ by means of the diaphorase–cytochrome–oxidase system. The experiments with acetaldehyde as substrate have furnished evidence that in yeast cells the reoxidation of $CoIH_2$ by means of this system is slower than the reoxidation by acetaldehyde as H_2 acceptor. This slower reoxidation in turn causes the decrease in the total amount of carbohydrate breakdown. Thus the Pasteur effect is in the last analysis due to the circumstance that under aerobic conditions part of the reduced cozymase persists in the reduced state thus diminishing the amount of acting cozymase.

Acknowledgments. The author is very much indebted to Mr. H. F. Holden for his interest in this work and to Mr. W. A. Rawlinson for the valuable assistance in the performance of the gas analyses and in the construction of parts of the apparatus. He gratefully acknowledges the courtesy of H. Hudson & Co. Pty. Ltd. and Effront Yeast Pty. Ltd. who presented the yeast for these experiments. He is also indebted to a grant from the E. M. Carty fund.

REFERENCES.

Ball, E. G. (1939): Bull. Johns Hopkins Hosp., 65, p. 253.
Bumm, E. *et al.* (1934): Z. physiol. chem., 223, p. 207.
Cook, S. F. (1930): J. gen. Physiol., 14, p. 55.
Cori, G. T. and Cori, C. F. (1936): Proc. Soc. exp. Biol. Med., 34, p. 702.
Cori, G. T. and Cori, C. F. (1937): *Ibid.*, 36, p. 119.
Cori, G T. and Cori, C. F. (1938): J. biol chem., 128, p. 375.
Cori, G. T. and Cori, C. F. (1940): *Ibid.*, 135, p. 733.
Dakin, H. D. (1914): *Ibid.*, 18, p. 91.
Dewan, J. G. and Green, D. E. (1938): Biochem. J., 32, pp. 626 and 1200.
Dixon, K. C. (1935): *Ibid.*, 29, p. 973.
Dixon, K. C. (1937): Biol. rev. Cambridge Phil. Soc., 12, p. 431.
Dixon, M. *et al.* (1937): Biochem. J., 31, p. 1347.
Euler, H. and Johansson, D. (1912): Z. physiol. Chem., 76, p. 347.
Euler, H. (1914): *Ibid.*, 89, p. 337; *Ibid.*, 90, p. 355.
Euler, H. *et al.* (1938): *Ibid.*, 252, p. 31; Naturwissenschaften, 26, p. 676.
Euler, H. *et al.* (1939): *Ibid.*, 258, p. 47.
Fink, H. and Lechner, R. (1940): Biochem. Z., 304, p. 425.
Flock, E. V. and Bollman, J. L. (1940): J. biol. Chem., 136, p. 469.
Genevois, L. (1927): Biochem. Z., 186, p. 461; *Ibid.*, 191, p. 147.
Gordon, A. H., Green, D. E. and Subrahmanyan, V. (1940): Biochem. J., 34, p. 764.
Gottschalk, A. (1923): Biochem. Z., 140, p. 348.
Gottschalk, A. (1925): The carbohydrate metabolism in animal cells; C. Oppenheimer's Handbuch der Biochemie des Menschen und der Tiere 2 Aufl., vol. II, p. 485 (Jena).
Guillemet, R. and Leroux, H. (1936): Compt. rend, soc. biol., 121, p. 465; 125, p. 903.
Hahn, A. *et al.* (1931): Z. f. Biol., 91, p. 53.
Harden, A. (1932): Alcoholic fermentation, London.
Harden, A. and Young, W. J. (1904): Ber., 37, p. 1052.
Heicken, K. (1938): Ann., 534, p. 68.
Jodlbauer (1888): Z. f. Rübenzuckerindustrie, 25, p. 308.
Kiessling, W. (1939): Biochem. Z., 302, p. 50.
Kostytschew, S. (1914): Z. physiol. Chem., 89, p. 367.
Krebs, H. (1928): Peterfi, Methoden der wissenschaftlichen Biologie, vol. II, p. 1048 (Berlin).
Kruyk, K. and Klingmüller, V. (1939): Biochem. Z., 300, p. 343.
Laser, H. (1937): Biochem. J., 31, p. 1671.
Leiner, G. (1935): Biochem. Z., 226, p. 186.
Lindner, P. (1919): Z. f. techn. Biol., 7, p. 68.
Lipmann, F. (1933): Biochem. Z., 265, p. 133; *ibid.*, 268, p. 205.
Lundin, H. (1923): *Ibid.*, 141, p. 310.
Lundsgaard, E. (1932): *Ibid.*, 250, p. 61.
Macfarlane, M. G. (1937): J. chem. Ind., 56, p. 935.
Mayer, P. (1923): Biochem. Z., 136, p. 487.
McAnally R. A. and Smedley-Maclean, J. (1935): Biochem. J., 29, p. 1872.
McAnally, R. A. and Smedley-Maclean, J. (1937): *Ibid.*, 31, p. 72.
Meyerhof, O. (1920): Arch. ges. Physiol., 185, p. 11.
Meyerhof, O. (1921): *Ibid.*, 188, p. 114; *ibid.*, 191, p. 128.
Meyerhof, O. (1922): *Ibid.*, 195, p. 22.
Meyerhof, O. (1924): *Ibid.*, 204, pp. 295, 448.
Meyerhof, O. (1925): Biochem. Z., 162, p. 43.
Meyerhof, O. and Finkle, P. (1925): Chemie der Zelle und Gewebe, 12, p. 157.

Myrbäck, K. (1936): Svensk kem. tidskr., 48, p. 55; Biochem. Z., 288, p. 329.
Needham, J. and Dixon, K. C. (1937): Biochem. J., 31, p. 1196.
Negelein, E. and Broemel, H. (1939): Biochem. Z., 301, p. 135; *ibid.*, 303, p. 132.
Negelein, E. and Wulff, H. J. (1937): *Ibid.*, 293, p. 351.
Neuberg, C. and Kerb, J. (1914): *Ibid.*, 58, p. 158.
Neuberg, C. and Gottschalk, A. (1924): *Ibid.*, 146, p. 164.
Pasteur, L. (1861): Bull. de la soc. chimique de Paris, 28th of June, pp. 79–80.
Schaeffner, A. and Specht, H. (1939): Naturwissenschaften, 27, p. 195.
Shibata, K. and Tamiya, H. (1930): Acta phytochemica, 5, p. 22.
Smedley-Maclean, J. (1922): Biochem. J., 16, p. 370.
Steiner, A. and Cori, C. F. (1935): Science, 82, p. 422.
Stern, K. G. (1940): Ann. Rev. Biochem., 9, p. 1.
Stier, T. J. B. (1935): J. gen. Physiol., 19, p. 339.
Stier, T. J. B. and Stannard, J. N. (1935): *Ibid.*, 19, p. 479.
Warburg, O. (1914): Ergebn. d. Physiol., 14, p. 263.
Warburg, O. *et al.* (1924): Biochem. Z., 152, p. 309.
Warburg, O. (1926a): *Ibid.*, 172, p. 432.
Warburg, O. (1926b): *Ibid.*, 177, p. 471.
Warburg, O. *et al.* (1939): *Ibid.*, 303, p. 40.
Widmark (1922): *Ibid.*, 131, p. 475.
Wieland, H. and Claren, O. B. (1932): Ann., 492, p. 183.
Wieland, H. and Sonderhoff, R. (1932): *Ibid.*, 499, p. 213.
Wieland, H. and Wille, F. (1933): *Ibid.*, 503, p. 70.
Willstaetter, R. and Rohdewald, M. (1937): Z. physiol. Chem., 247, p. 269.
Wind, F. (1926): Klin. Wochenschr., 30, No. 5.
Winzler, R. J. and Baumberger, J. P. (1938): J. cellular comp. physiol., 12, p. 183.

RELATIONS BETWEEN PROTEIN-NITROGEN, PROTEIN-SULPHUR AND CHLOROPHYLL IN LEAVES OF SUDAN GRASS

by E. A. HANSON,[1] B. S. BARRIEN[2] AND J. G. WOOD

(From Department of Botany, University of Adelaide, South Australia).

(*Accepted for publication 30th April, 1941.*)

In an earlier paper Barrien and Wood (1939) showed that the ratio protein-N/protein-S in leaves decreased in value during the life cycle of Sudan Grass (*Andropogon sudanensis*, L. and B.) and ascribed this decrease to more rapid loss of sulphur-poor protein compared with a sulphur-rich protein. In this paper we have attempted to substantiate this conclusion by isolating and analysing chloroplast-protein. In addition, we have related amounts of protein to amounts of chlorophyll throughout the life cycle.

EXPERIMENTAL.

Treatment. Seeds of a pure line of Sudan Grass were sown in pots containing 14 kg. of a mixture of loam and sand (2: 1). One week after planting each pot received the following nutrients: 0·4 gm. $NaNO_3$; 0·6 gm. KH_2PO_4; 2·0 gm. K_2SO_4; 0·4 gm. $MgSO_4.7H_2O$; 0·74 gm. $CaCl_2$; 0·06 gm. $FeCl_3$; 0·0016 gm. $MnSO_4.4H_2O$. Sixty pots were prepared and 30 seeds sown in each; after Harvest 1 these were reduced to 5 even plants per pot. The pots were kept in a glass house and the water content of the soil maintained at approximately 60 p.c. saturation.

Seeds were planted on 26th February, 1940, and harvests made in early mornings 23, 35, 51, 58, 71 and 85 days after planting (Series I). Originally it was intended to study the effect of additional N-supply and to this end 1 gm. $(NH_4)_2SO_4$ per pot was added 24 hours before harvesting to similarly prepared pots (Series II). This time was insufficient to bring about any significant change in amounts of protein or chlorophyll so that Series II serves as a replicate to Series I. Chloroplast estimations were not made in Series II. In both cases exsertion of inflorescences commenced on 24th April and exsertion of stamens on 4th May.

Chloroplast isolation. Chloroplasts were isolated and purified by fractional centrifugation using the method already described by Hanson (1941). At each harvest chloroplasts were obtained from duplicate batches of 100 gm. fresh leaves. The final collection no longer contained contaminating material and consisted of bright glistening whole chloroplasts. Further centrifugations could not be performed since, about 4 hours after grinding, the chloroplasts suddenly assume a dull appearance and disintegrate. It proved impossible to isolate chloroplasts from yellowing leaves in late stages of the plants' development since they disintegrate rapidly after grinding.

The bulk of chloroplasts was divided into two portions: one for N-estimation and one for S-estimation.

Chlorophyll. The pigments were extracted with 90 p.c. acetone. After removal of the carotinoids, the chlorophyll was estimated colorimetrically as ethyl chlorophyllide, using as standard a sample of the pure substance prepared according to Hanson (1939). The chlorophyll content of aliquots of the fresh leaf material was also determined by the same method.

Chloroplast-Protein. The residue from the acetone extraction was ground with 4 p.c. tannin solution and then filtered. Nitrogen was estimated on the residue by micro-Kjeldahl and sulphur after explosion in a calorimeter bomb by the method described by Barrien and Wood (1939).

Total protein was determined in leaf material rapidly dried in an air stream at 90° C. and then finely ground. Aliquots were suspended in distilled water and the pH adjusted to 4·5 with trichloracetic acid using a glass electrode. The suspension was warmed at 40° C. for 15 mins., brought to the boil for 1 min., stood overnight and filtered. Nitrogen and sulphur were determined in the residue by the methods described above.

Assimilation rates. An attempt was made to determine rate of carbon assimilation of leaves at each harvest using Blackman's palladium apparatus (Briggs, 1920). The conditions were: CO_2, 5 p.c.; light intensity, about 18,000 metre candles; temperature, 25° C. Unfor-

[1] Research Officer under Federal Government Research Grant to Universities.

[2] Research Assistant under Federal Government Research Grant to Universities.

tunately the material proved sensitive to exposure to hydrogen and the assimilation rate quickly rose to a maximum value and thereafter slowly declined to zero within about 4 hours. We have reported the maximum value obtained in each case.

Results. All analyses were made on duplicate samples and mean values are presented. The results are presented on an absolute basis (mg. of substance per plant) for Series I in Fig. 1 and on a relative basis (percentage of dry weight) for both series in Table 1.

TABLE 1.

Harvest.	Days after planting.	Mean dry weight per plant.	Assimilation rate.	Chlorophyll.	Total protein-N.	Chloroplast protein-N.	Total protein-S.	Chloroplast protein-S.	Total protein-N. / Total protein-S.	Chloroplast protein-N. / Chloroplast protein-S.	Chloroplast protein-N. / Chlorophyll.
Series I.											
1	23	0·24	50·2	1·04	4·28	0·30	0·264	0·044	16·2	6·7	0·28
2	35	0·88	38·1	1·88	3·61	1·40	0·207	0·201	17·4	7·0	0·75
3	51	2·40	15·0	1·61	2·54	0·91	0·167	0·109	15·2	8·3	0·57
4	58	3·46	11·9	1·51	2·20	0·90	0·140	0·125	15·7	7·2	0·60
5	71	3·63	11·0	0·93	1·64	0·74	0·113	0·103	14·5	7·2	0·80
6	85	3·57	—	0·64	1·17	—	0·088	—	13·3	—	—
Series II.											
1a	not harvested separately.										
2a	36	0·89	37·0	1·85	3·28	1·34	0·201	0·201	16·3	6·7	0·72
3a	52	2·70	15·9	1·40	2·68	0·93	0·167	—	16·0	—	0·66
4a	59	3·28	—	—	2·04	—	0·135	—	15·1	—	—
5a	72	3·30	9·8	1·07	1·92	—	0·129	—	14·9	—	—
6a	86	3·56	—	0·60	1·12	—	0·086	—	13·0	—	—

Dry weight in gm. per plant.
Assimilation rate in ml. CO_2 per gm. dry weight per hour.
Other substances in gm. per 100 gm. dry material.

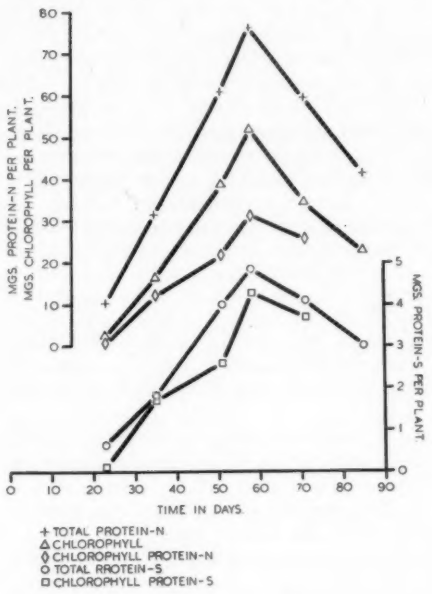
DISCUSSION.

Protein-N and Protein-S. Fig. 1 shows that the absolute amounts of total protein-N and protein-S attain maxima at Harvest 4 (a time practically coincident with inflorescence development), and thereafter decline. Amount of chlorophyll also attains its maximum at the same time; the slopes of the curves approximate closely that for total protein-N, but during late senescence chlorophyll disappears more rapidly than does total protein-S. Chloroplast protein-N and protein-S attain their maxima at the same time as the total protein; this would appear to be a general phenomenon since Granick (1938b) reports a similiar coincidence in tobacco.

In young leaves (Harvest 1) the chloroplast protein-N represents only a small fraction (about 7 p.c.) of the total protein-N, whilst in older leaves from 35-40 p.c. of the total protein-N is localized in the chloroplasts. When protein-S is used as a basis for comparison the same general effect is observed but 70 p.c. or more of the protein-S is localized in the chloroplasts of older leaves. The constancy of the ratio chloroplast protein-N/chloroplast protein-S suggests that a single protein, or a group of allied proteins, is restricted to the chloroplast.

The ratio total protein-N/total protein-S has a higher value throughout, indicating the relative richness of chloroplast proteins in S, but shows a continuous decline in value throughout the life cycle of the plant. The decline in value of this ratio confirms earlier observations with this plant reported by Barrien and Wood (1939). In the latter paper we ascribed the decrease in ratio to the more

rapid decrease in a sulphur-poor protein compared with a sulphur-rich protein. The sulphur-rich protein appears to be the chloroplastic protein.



Chlorophyll and protein. Table 1 shows that the ratio protein-N/chlorophyll does not remain constant throughout the life cycle. This is especially evident in young leaves at Harvest 1 when chlorophyll is present in relatively high amount compared with chloroplast-protein. Later a marked increase in chloroplast-protein and also in chlorophyll occurs although total protein is already beginning to decline in amount per unit dry weight. The data indicate that up to the time of maximum content per plant chlorophyll expressed on a dry weight basis decreases less rapidly than does chloroplast protein—though changes in the ratio are small—but during senescence chlorophyll disappears more rapidly than does chloroplast-protein. The rate of assimilation falls in value throughout the life cycle and is apparently unrelated to content of either chlorophyll or chloroplast-protein. The fall in rate is more closely associated with that of total protein than with any other component determined.

Recent work (see Weier, 1938) has focussed attention on the idea that chlorophyll forms part of a pattern within the chloroplast. Various speculations (e.g., Hubert, 1935 and Frey-Wyssling, 1937) have been brought forward but, in general all suggest that hydrophilic porphin nuclei are in contact with protein molecules whilst the lyophilic phytol chain is associated with lipoids. From analogy with other chromoproteins (haemoglobin, catalase, cytochrome *c*) in which four porphin nuclei are associated with a globin of molecular weight 68,000, it has been suggested (Hubert, 1935; Hanson ,1939) that four chlorophyll molecules are associated with a globin carrier of similar molecular weight. Such a combination would yield a protein/chlorophyll ratio of 18·5. Mommaerts (1938) has attempted to isolate grana from chloroplasts by disintegration and centrifugation and reports a protein/chlorophyll ration of 18·2. He obtained different values under different conditions; and we would state that we have been unable to obtain grana which are not highly contaminated with cytoplasmic fragments. These might account for the high ratios obtained by Mommaerts.

Plant globins usually contain about 16 p.c. N; using this figure to calculate protein and assuming a molecular weight of 68,000, our data indicate that about 40 molecules of chlorophyll are associated with protein in the plastid at Harvest 1 and from 15-20 molecules of chlorophyll with each protein molecule at the other four harvests. These figures are in close agreement with those of Granick (1938b) who estimated that in tomato approximately 30 chlorophyll molecules were associated with each protein of molecular weight 100,000 (i.e. about 20 chlorophyll molecules per protein of molecular weight 68,000). They also agree closely with

those reported by Hanson (1941) for *Phalaris* (16 chlorophyll molecules per protein carrier of M.W. 68,000) and by Smith (1940) for spinach and aspidistra (12 chlorophyll molecules per protein carrier of M.W. 68,000). It is possible that some variation occurs between species.

The existence of such a number of chlorophyll molecules per protein carrier is within physical limits. Hanson (1939) showed that the chlorophyll molecule is a flat plate whose surface area in the plane of the carbon skeleton is 242Å^2. The total surface of a globin molecule of molecular weight 68,000 is approximately 5000Å^2. It is apparent therefore that at least twenty molecules could adopt even a horizontal position on the surface of the protein without touching one another.

We would point out further that chlorophyll is localized in the grana of the chloroplast whilst presumably protein occurs throughout the stroma as well. In this case a greater number of chlorophyll molecules than we have suggested would be associated with one protein molecule.

SUMMARY.

Pure chloroplasts were isolated from leaves of a pure line of Sudan Grass (*Andropogon sudanensis*, L. and B.) at intervals during the life cycle. Analyses of both whole leaf and chloroplast material were made and the following conclusions drawn:

Total protein-N, total protein-S, chlorophyll, chloroplast protein-N and chloroplast protein-S attain a coincident maximum during the life cycle and thereafter decrease in amount.

In young leaves chloroplast protein comprises about 7 p.c. of the total protein, but later 35-40 p.c. of total protein is localized in the chloroplast.

The chloroplast proteins are rich in sulphur and contain approximately 70 p.c. of the total protein-sulphur.

The ratio chloroplast protein-N/chloroplast protein-S remains approximately constant during the life cycle, but the ratio total protein-N/total protein-S decreases in value indicating greater utilization of cytoplasmic proteins during senescence.

In young leaves chloroplasts are relatively high in chlorophyll but low in protein; later there is a marked increase in chloroplast-protein; thereafter both chlorophyll and protein decrease in amount.

During senescence chlorophyll disappears from the leaves more rapidly than protein.

Assuming a molecular weight of 68,000 for protein, about 20 chlorophyll molecules are associated with each protein molecule in the chloroplast over the greater part of the life cycle of the plant.

Acknowledgments. We are indebted to the Director of the Waite Agricultural Research Institute, University of Adelaide, for glass-house facilities.

REFERENCES.

Barrien, B. S. and Wood, J. G. (1939): New Phytol., 38, p. 257.
Briggs, G. E. (1920): Proc. Roy. Soc. Lond. B. 91, p. 249.
Frey-Wyssling, A. (1937): Protoplasma, 28, p. 279.
Granick, S. (1938a): Amer. J. Bot., 25, p. 558.
Granick, S. (1938b): *Idem.*, p. 561.
Hanson, E. A. (1939): Rec. Trav. bot. néerl., 36, p. 183.
Hanson, E. A. (1941): Austral. J. exp. Biol., 19, p. 157.
Hubert, B. (1935): Rec. Trav. bot. néerl., 32, p. 323.
Menke, W. (1938): Z. f. Bot., 32, p. 273.
Mommaerts, W. F. H. M. (1938): Kon. nederl. Akad. Wet., 61, p. 3.
Smith, E. L. (1940): Science, 91, p. 199.
Weier, E. (1938): Bot. Rev., 4, p. 497.

INFECTIONS OF THE RESPIRATORY TRACT OF CHICK EMBRYOS BY THE VIRUSES OF PSITTACOSIS AND INFECTIOUS LARYNGOTRACHEITIS. A COMPARISON WITH INFLUENZA VIRUS INFECTIONS

by F. M. BURNET AND M. FOLEY[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(*Accepted for publication 14th May, 1941.*)

During the past year we have been actively interested in the phenomena associated with infection of the chick embryo by the amniotic route with various strains of influenza virus; a number of papers have been published (Burnet, 1940; Burnet and Foley, 1940, 1941). Most of this work has been concerned with matters of potential practical importance, notably the use of the method for the primary isolation of virus and for the production of virus vaccines of graded virulence. At the same time we have had in mind the possibility that this technique might contribute something of value to the understanding of the tissue specificity of viruses. As opportunity offered we have tested the different viruses available in this laboratory for their power to infect by the amniotic route. In most instances we have made only limited tests and have accepted the evidence of a tracheal fluid free from cells as indicating failure of the virus concerned to "take" by this route. On this criterion vaccinia, herpes, fowl-pox, pigeon-pox, louping-ill and Rous sarcoma viruses, all in the form of chorioallantoic membrane material, failed to infect. The viruses of psittacosis and of infectious laryngotracheitis of fowls have, however, produced definite lesions. Relatively few experiments have been done with laryngotracheitis virus and the results must be regarded as only of a preliminary character. A more extensive series of experiments with psittacosis virus has been carried out, including maintenance of the virus for over 30 passages by the amniotic route.

MATERIAL AND METHODS.

Laryngotracheitis virus. An old stock strain of this virus failed to infect (Burnet, 1940) and the present work has been carried out with a strain derived from dried tracheal exudate kindly sent us by Mr. L. Hart of Glenfield Veterinary Research Station. New stock chorioallantoic material was prepared from this strain and with this definite amniotic infections were produced.

Titrations were made by the standard chorioallantoic method.

Psittacosis virus. The strain used P. was isolated in 1936 from sputum of a patient severely ill who had been infected from a "smoker" parrot (Barnardia). It was the most virulent strain for mice which we have obtained in Australia. The same strain was used in the work by Rudd and Burnet (1941) and amniotic passage was commenced with a substrain that had been passaged on the chorioallantois for 76 generations. In the process it had undergone a slight diminution in virulence for mice though still giving typical peritoneal lesions.

Titrations of psittacosis virus were made by intranasal inoculation in mice according to the technique of Rudd and Burnet (1941). Serial dilutions are inoculated intranasally in 0·05 c.c. amounts and the number of foci produced observed after eight days.

Amniotic inoculations. With both viruses the same technique was used as has been described for influenza virus experiments (Burnet, 1940a, b). Approximately 0·05 c.c. of inoculum is placed in the amniotic cavity of embryos at the twelfth day of incubation. The inoculated eggs are held at 35° C. and examined usually three days after inoculation. Smears of the tracheal fluid are prepared and stained with Leishman's stain for cellular detail and in the case of psittacosis infections by Castaneda's method for L.C.L. bodies. Material for subinoculation or for titration of virus content was usually the pooled allantoic and amniotic fluids. All fluids were cultured on blood agar to ensure absence of bacterial contamination.

[1] This work has been carried out under grants for research on virus diseases from the National Health and Medical Research Council and from Mr. E. Alec Cato.

1. *Laryngotracheitis Virus Infections.*

Lesions produced by laryngotracheitis virus. After incubation for three days it is usual to observe some foci on the chorioallantois near the point of inoculation and sometimes the amnion is thickened with specific lesions. The amniotic fluid is usually turbid but the amount is more or less normal. The disappearance of all free amniotic fluid which is so often seen with influenza virus infections is never observed. On a few occasions distinct lesions of the larynx have been visible with a lens in the form of nodular irregularities but usually nothing definite can be seen. The tracheal fluid is turbid and may show flakes of epithelium in addition to a fine turbidity due to discrete cells. The lungs are not macroscopically altered.

In smears of tracheal fluid stained by Leishman's method leucocytes are the dominant cells. They are usually fairly mature forms with bilobed nucleus and eosinophil granules and contrast with the less mature forms characteristic of influenza virus infections. Epithelial cells are also present and show variable degrees of pathological change. Vacuolation of the cytoplasm is frequent and many cells are indistinguishable from the similar type found in influenzal infections. Many show nuclear damage but probably because of the method of fixation and staining used the changes could not be recognized as corresponding to the intranuclear inclusions visible in lung sections. In some smears a striking feature has been the presence of large cells apparently of epithelial origin actively phagocytic for red cells and containing large basophilic granules which appear to be nuclear fragments.

Lung sections fixed in formol saline and strained with haematoxylin and eosin show lesions which are usually limited to the larger bronchial divisions. The lesions are of the same general character as those observed in the natural disease of the fowl without of course the inflammatory changes due to secondary bacterial infection. In the larger bronchi (with cartilage sheath) and the trachea there is little evidence of proliferation; the cells remain more or less normally arranged but show the characteristic nuclear changes and variable degrees of degeneration and vacuolation. Eventually there is desquamation of the infected area. In none of the sections examined was there a uniform involvement of the epithelial lining; occasionally there would be practically complete desquamation of epithelium in an infected bronchus but elsewhere only a small group of cells would show intranuclear inclusions with the others in the section apparently normal.

In the smaller bronchial divisions a characteristic feature, seen sometimes also in the larger bronchi, is the appearance of buds of close packed cells all the nuclei of which show typical inclusions. These buds soon become partly necrosed and may be thrown off almost intact into the lumen.

Although most sections show very little change in the parabronchi several sections have contained an isolated group presumably supplied by a single secondary bronchus in which characteristically infected cells occur. One lung from an embryo infected three days earlier showed extensive involvement of nearly all the parabronchi. Figures 1 and 2 show characteristic fields at high and low magnifications. It will be seen that the picture differs sharply from that produced by influenza virus, proliferation being more evident and necrosis and desquamation much less prominent. There is practically no real disorganization of structure and the pathological changes are strictly confined to the respiratory epithelium. In the affected region practically every nucleus shows characteristic alteration sometimes with classical inclusion-body formation, more often with a peripheral chromatin ring and the whole centre filled with uniformly staining amorphous material. The tracheal smear from this embryo was unusual in the very large proportion of epithelial cells it contained. Many of these were in the form of almost intact buds. The nuclei showed various degrees of damage but none of the appearances at all closely resembled the intranuclear inclusion as seen in formalin-fixed material stained with haematoxylin.

Multiplication of virus by amniotic route. The activity of laryngotracheitis virus is much lower than that of influenza strains given by the same method and we have obtained no evidence of any increase in virulence with passage. Embryos showing typical tracheal smears and histological findings after three or four days' incubation provide fluids which produce small numbers of foci when diluted $1:10^3$ or $1:10^4$ i.e. they contain approximately 2×10^4 to 6×10^5 infective units per c.c. In nearly every series of eggs, however, there is a proportion of embryos which show no definite tracheal fluid changes and a passage series can be maintained only by using selected embryos. The longest passage series we have obtained was for nine generations. It is possible that some of the difficulties of passage are due to the existence of eggs from fowls which are immune to laryngotracheitis as a result of subclinical infection. It is known that there is no obvious disease in the farm from which the eggs are obtained but in view of our earlier experience (Burnet, 1936) there remains a possibility of subclinical infection. Under present circumstances it has been impossible to make the extensive investigations which would be necessary to confirm or refute this suggestion.

2. *Psittacosis Virus Infections.*

A. *Behaviour of a strain mixed with influenza virus.* Our first experiments were made with an egg passage strain that had been transferred on the chorioallantois of nine to ten day embryos for 76 generations. Subsequent studies indicated that somewhere between the 30th and 76th

Fig. 1. Laryngotracheitis infection of chick embryo lung. All the parabronchi visible in the area are heavily infected but there is no disorganization of the normal arrangement.

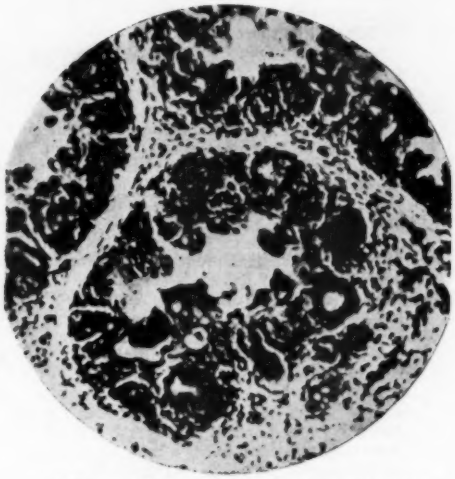


Fig. 2. High power view of an area near the centre of Fig. 1 to show irregular proliferation of the parabronchial epithelium.

Fig. 3. Typical influenza virus lesion at same magnification as Fig. 1. There is far greater epithelial damage and inflammatory reaction than with laryngotracheitis infections.

passage this strain had become contaminated with an influenza virus apparently one of the strains isolated locally during 1939 or 1940. The lesions produced were immediately recognized as being strikingly like those of influenza virus and the possibility of contamination considered. Psittacosis virus was easily demonstrated by the presence of L.C.L. bodies in smears and by the characteristic foci produced after intranasal inoculation in mice. These mice showed no visible influenza virus lesions. As a precaution the virus was passaged once more on the chorioallantois with immune influenza ferret serum and as the influenza-like lesions continued to appear in eggs inoculated amniotically it was thought that the psittacosis virus had probably developed an increased virulence for embryonic tissues in the course of passage on the chorioallantois. During the subsequent passage series a number of virus titrations were made by the intranasal method in mice without the appearance of any suspicious lesions. In these titrations the lowest dilution normally used was 1: 10^4, so that weak influenza virus would not be expected to produce lesions at the dilutions examined.

The existence of the contaminating virus was made known by the results of an experiment in which the virus content of the allantoic fluids from several infected embryos was being determined by intranasal inoculation in mice. No psittacosis foci appeared but typical areas of influenza virus consolidation. Appropriate experiments showed that the contaminant was an influenza virus of "Melbourne" antigenic type, of low ferret pathogenicity, producing weak lesions on the chorioallantois and of rather low pathogenicity for mice. Psittacosis virus free from the contaminant was obtained by two passages with an excess of ferret immune serum through mouse lungs.

This accidentally contaminated series had been maintained for 31 passages before the influenza virus was detected. During the period the two viruses had apparently both multiplied freely. In the earliest passages L.C.L. bodies were rare but after the tenth passage they became more numerous and in all subsequent passages they were plentiful in both lung smears and smears of the tracheal exudate. In some lung sections large numbers of cells infected with L.C.L. bodies could be seen in the parabronchi, the infection being more intense than any we have seen with psittacosis virus alone. A point of particular interest was observed in comparative titrations of mixed allantoic and amniotic fluids in mice and by the amniotic route. Despite the regularity of the focal counts in the mouse lung the titre in the eggs was very much higher. Table 1 shows the results of titrating 27th and 31st passage material.

Rough calculations from the number of L.C.L. bodies visible in stained smears indicated that if each particle was active the infective fluids should titrate to 10^8 or higher. It is clear therefore that despite the regularity of the counts of foci in the mouse lung only a small proportion of the virus particles viable by the more sensitive test were capable of inducing mouse lung foci. For reasons to be described later experiments of this type with pure psittacosis virus were difficult to interpret and our chief reason for reporting the work with the contaminated virus is to make use of the data in Table 1.

TABLE 1.

Comparative titrations of embryonic fluids in mice and eggs.

		Amniotic titration.	Foci in lungs of mice.		
27th passage	10^{-4}	+ L, +, (++)	19	14	11
	10^{-5}	+ L, + L, (+)	2	0	0
	10^{-6}	+ L, (−), − (+)	1	0	0
	10^{-7}	+ L, + L, + L, −			
	10^{-8}	−, −			
31st passage	10^{-4}	+ L, + L	16	14	14
	10^{-5}	+ L, ± L, (+)	4	2	1
	10^{-6}	+ L, + L, + L	0	0	0
	10^{-7}	+ L, + L			
	10^{-8}	+ L, + L, −			

+ positive tracheal smear. L L.C.L. bodies seen. (+) death of embryo presumably from specific infection. ± very few cells in tracheal smear. − embryo normal at four or five days. (−) embryo normal when examined at three days.

B. *Lesions produced by psittacosis virus alone.* With the same strain of virus from which the contaminant had been eliminated continued passage could be readily maintained but the lesions were much less severe. With large inocula a proportion of embryos died usually about the fourth day. In surviving infected embryos examined at this period the embryo usually appeared somewhat underdeveloped and pale, the amnion was never contracted down on the embryo in the way characteristic of influenza virus infections. The tracheal fluid was sometimes heavily turbid but much more usually there was only a slight background turbidity with a few larger flakes. The lungs were somewhat paler than normal. Smears from the tracheal fluid showed large numbers of L.C.L. bodies but the cellular content was much less than in influenza virus infections. The more turbid fluids contained numerous leucocytes, some large pale epithelial

cells containing L.C.L. bodies and many almost normal epithelial cells. In almost all smears the great majority of the L.C.L. bodies were extracellular. Many positive smears contained only debris with a few almost normal cells but fair numbers of L.C.L. bodies.

Sections of lungs taken three or four days after inoculation usually appeared almost normal under the low power and there was never gross disorganization of the lung structure. The most characteristic lesions were in the epithelium of the larger bronchi. A proportion of the superficial cells was pale and swollen and contained large numbers of L.C.L. bodies easily visible in haematoxylin and eosin stained sections. The infected cells occurred singly or in small groups and almost without exception were in the most superficial layer of epithelium. There was very little disturbance of the normal cellular arrangement and no infiltration with inflammatory cells. Lesions of this sort were present in every section which contained a suitable bronchus for examination. The parabronchi were involved only in some sections. In these a proportion showed damaged cells free in the lumen and some disorganization of the normal pattern of saccule formation. Isolated cells with large numbers of L.C.L. bodies in the cytoplasm were occasionally seen. There was little evidence of any inflammatory or proliferative response around these cells.

In summary the process is essentially an infection of the superficial epithelial cells of the larger bronchial divisions. There is free multiplication within the infected cells and eventual destruction with liberation of virus into the lumen but there is strikingly little damage to adjacent cells and almost no inflammatory changes. The parabronchial epithelium is susceptible but the virus clearly has not the same power of rapid spread possessed by influenza virus and only a few parabronchi normally become infected.

Multiplication of virus and content of virus in the embryonic fluids. Subinoculations are normally made from amniotic fluid usually with a certain amount of allantoic fluid in addition. Table 2 shows the results of mouse lung titration of various fluids from embryos three and four days after infection. It will be seen that the virus content of the amniotic fluid is much higher than that of the allantoic fluid and also that the titre of virus in mixed amniotic and allantoic fluid is of the same order as was found with the mixed influenza-psittacosis infections in the first series as shown in Table 1. Titrations by the amniotic method with the pure virus were unsatisfactory owing to the difficulty of obtaining an endpoint with very dilute virus. Embryos inoculated with 10^{-3} and 10^{-4} dilutions of mixed amniotic and allantoic fluids giving approximately thirty foci at 10^{-4} in mice, showed no macroscopic lesions at five days and only one visibly turbid tracheal fluid. The other fluids contained only an occasional flake of debris yet when the small amount of sediment was smeared L.C.L. bodies were visible in all. The same fluid was tested by intraperitoneal inoculation of 0·5 c.c. of 10^{-2}, 10^{-4} and 10^{-6} dilutions in mice; all showed typical lesions.

TABLE 2.

Mouse lung titration of fluids from infected embryos.

Fluid.	Foci at dilutions shown.				Titre per c.c.
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
Amn. and all. 4 day	++	42, 40, 29	2, 2, 1	0, 0	6×10^6
„ „ „ 4 day	++, 50	6, 5, 4	1, 0, 0	0, 0, 0	1×10^6
	Und.	10^2	10^3	10^4	
Amn. 059 3 day.		†6, †6	70, 62, ++	0	$1{\cdot}3 \times 10^7$
All. 059 3 day	62	0, 0	0, 0		$1{\cdot}2 \times 10^3$
Amn. 060 3 day		++, ++, ++	30, 50±, ++	0, 0, 0	8×10^6
All. 060 3 day	†7, ++, ++	60±, 50±, 56	1, 1, 0		1×10^5

In order to determine whether the results obtained in amniotic inoculation were dependent on the long preceding period of chorioallantoic passage a few experiments were made with mouse passage virus. Mice were inoculated with reconstituted dried material intranasally and an emulsion from these lungs was used for amniotic inoculation. Only one of the twelve embryos inoculated showed definite tracheal smear findings (at four days). Titrations of the fluids from representative embryos opened at two, three and four days however showed that multiplication had taken place. The foci produced per c.c. were two day $2{\cdot}6 \times 10^3$, three day 1×10^4, 4×10^2, four day $2{\cdot}6 \times 10^3$. These figures are much lower than those obtained at the same time for fluids from embryos inoculated with amniotic passage virus and included in Table 2. In all probability the slight activity shown by this mouse passage material was due to the relatively low titre of the mouse lung emulsion used. With one further mouse passage inoculated embryos gave results very similar to those obtained with amniotic passage material the titres of fluids from two embryos at three days being both over 10^7 per c.c. as tested in the mouse lung.

DISCUSSION.

It is evident from these results that neither laryngotracheitis nor psittacosis virus has the power of spreading rapidly through the respiratory system of the

embryo which is shown by influenza virus. As might be expected the virus of laryngotracheitis produces more definite lesions and may involve much more of the respiratory epithelium than we have observed in any psittacosis infections. Without a much more extensive search for lesions in other organs which might be infected after amniotic inoculation we should not be justified in claiming that the experiments show any selective pneumotropism of psittacosis virus. In some ways the results in chick embryos and in mouse lungs are analagous. Psittacosis virus introduced into the respiratory tract of either organism readily induces foci of primary infection but there is no surface spread from these primary foci to involve the whole of the respiratory epithelium. With laryngotracheitis the foci observed in trachea and bronchi of the chick embryo are more extensive than those of psittacosis but only exceptionally do they spread to involve the whole of the parabronchi.

From the practical point of view the technique of amniotic inoculation offers no advantage over current methods of studying these two viruses.

SUMMARY.

The viruses of psittacosis and infectious laryngotracheitis of fowls will multiply and produce lesions when inoculated amniotically in chick embryos.

Laryngotracheitis virus gives rise to lesions of the trachea, bronchi and parabronchi which show the characteristic features of epithelial proliferation, formation of intranuclear inclusions and degeneration without much gross necrosis.

Passage of laryngotracheitis virus can be carried on for several generations but there are considerable numbers of embryos which appear to be resistant to infection.

Psittacosis virus multiplies freely but produces relatively minor lesions almost confined to superficial cells in the epithelium of the larger bronchi.

When mixed with a strain of influenza virus a strain of psittacosis virus persisted for over thirty passages. Evidence was obtained that in the presence of influenza virus the amnotic method was capable of detecting virus in considerably higher dilution than was effective in the mouse lung.

Amniotic fluid from embryos infected with psittacosis contained much more virus than allantoic fluid.

REFERENCES.

Burnet, F. M. (1936) : J. exp. Med., 63, p. 685.
Burnet, F. M. (1940) : Brit. J. exp. Path., 21, p. 147.
Burnet, F. M. (1940) : Austral. J. exp. Biol., 18, p. 353.
Burnet, F. M. (1941) : *Ibid.*, 19, p. 39.
Burnet, F. M. and Foley, M. (1941) : *Ibid.*, 19, p. 101.
Rudd, G. V. and Burnet, F. M. (1941) : Med. J. Austral., 1, p. 68.

THE INFLUENCE OF ADRENAL CORTICAL AND SEX HORMONES ON LIVER GLYCOGEN

by A. B. CORKILL AND J. F. NELSON

(From the Baker Institute of Medical Research, Alfred Hospital, Melbourne).

(*Accepted for publication 8th July, 1941.*)

Although there has been considerable controversy as to the "prepotent" function of the adrenal cortex, it now appears that of the various substances isolated no one can be regarded as the "vital" hormone. Thus desoxycorticosterone, most potent in respect of regulating electrolyte balance, has practically no effect on carbohydrate metabolism.

The subcutaneous injection of cortical extracts and certain of the pure cortical hormones into rats and mice leads to a deposition of liver glycogen (Long *et al.*, 1940); (Grattan and Jensen, 1940).

In the following experiments the influence of cortical extracts, desoxycorticosterone and sex hormones on liver glycogen and liver fat has been studied in young rabbits.

METHODS.

Young English hutch rabbits from 6–8 weeks old were used. The substances to be tested were injected subcutaneously in two equal quantities, the first injection being at 5 p.m. when food was withdrawn from the cages; at 9 a.m. the next morning a second injection was given. At 3 p.m. the animals were killed and bled out by severing the main blood vessels of the neck. For the estimation of liver fat (total lipoid), 2 gm. of minced liver were washed into a 100 ml. volumetric flask with 50 ml. of 95 p.c. alcohol. The flask was then placed in the boiling water bath until the alcohol began to boil. After cooling, 30 ml. of ether were added and the contents made up to volume with alcohol. Following filtration, 50 ml. of filtrate were evaporated to dryness in a tared Erlenmeyer flask. Liver glycogen was estimated by the method of Evans *et al.* (1931).

RESULTS.

The results are shown in Table 1 and for the purpose of comparison the effects produced by anterior pituitary extracts are included. The latter were either crude saline extracts (C.S.E.) or saline extracts prepared from an acetone-dried powder (Ac.P.). In both types 2 ml. of extract were equivalent to 1 gm. of fresh gland. The cortical extract used was "Eschatin" (Parke-Davis), and we are indebted to the Australian representative of Messrs. "Ciba" for generous supplies of sex hormones and desoxycorticosterone.

TABLE 1.

Nature and total amount of substance injected.	Average rabbit weight. gm.	Average liver weight. gm.	Average liver fat. p.c.	Average liver glycogen. p.c.	No. of animals. used.
Nil, Normal controls	895	26	4·4	0·45	10
Anterior pituitary extract (C.S.E.), 2 gm.	890	48	12·2	3·2	8
Anterior pituitary extract (Ac.P.), 2 gm.	916	42	11·7	3·0	10
Cortical extract, 1 ml.	898	25	3·9	4·0	8
Desoxycorticosterone, 5 mg.	900	24	4·0	0·5	6
Testosterone, 5 mg.	848	26	3·5	0·45	6
Progesterone, 5 mg.	900	22	4·2	2·5	7
Oestradiol dipropionate, 5 mg.	916	24	4·5	0·5	8

The results show that in young rabbits the subcutaneous administration of anterior pituitary extract, adrenal cortical extract and the hormone of the corpus luteum leads to a deposition of

liver glycogen. Unlike anterior pituitary extract, the latter two hormones do not cause an increase in liver weight or liver fat. Grattan and Jensen (1940) consider that only those adrenal cortical hormones with a keto or hydroxyl group at C_{11} exert an influence on carbohydrate metabolism. We agree with this contention in respect of desoxycorticosterone, but our positive results with progesterone are not in accord. In this connection it is interesting to note that Gaunt *et al.* (1939) found that the injection of large quantities of progesterone (50 mg.) into ferrets caused an increase in liver glycogen. No effect was found in rats.

The effect of anterior pituitary extracts on liver fat and glycogen in adrenalectomized animals. McKay and Barnes (1937) have shown that anterior pituitary extracts potent in respect of producing a deposition of fat in the livers of rats, fail to do so in adrenalectomized animals. We have repeated these experiments in rabbits and in addition have estimated liver glycogen. Adrenalectomy was performed as described in a previous paper (Corkill and Ennor, 1940). The remarks previously made concerning the survival of adrenalectomized rabbits without the administration of cortical extract etc. apply also to the present experiments. The animals used were semi-adults. The anterior pituitary extract was prepared from acetone-dried glands. From a large number of observations we were certain that extracts prepared from this particular batch of powder would invariably promote a deposition of liver fat and glycogen. In view of this the results shown in Table 2, although obtained on only a few animals, must be regarded as significant.

TABLE 2.

Type of animal.	Treatment.	Rabbit weight gm.	Liver weight gm.	Liver fat p.c.	Liver glycogen p.c.
Normal fasting control	—	1,040	31	4·5	0·5
Adrenalectomized control	—	960	28	4·0	0·3
Adrenalectomized	A.P. extract	1,090	47	15·0	0·6
Adrenalectomized	A.P. extract	1,400	65	16·4	0·8
Fasting normal	A.P. extract	960	41·5	11·6	2·5
Fasting normal	A.P. extract	980	49·5	14·4	2·8

SUMMARY.

The subcutaneous administration of adrenal cortical extract and progesterone to young rabbits causes a deposition of liver glycogen. Testosterone, oestradiol and desoxycorticosterone, tested under similar conditions, were inactive.

Anterior pituitary extract administered to adrenalectomized rabbits produces a deposition of liver fat, but, in contrast to its action in normal animals, it fails to promote glycogen formation.

REFERENCES.

Corkill, A. B. and Ennor, H. (1940): Austral. J. exp. Biol., 18, p. 379.
Evans, C. L., Tsai, C. and Young, F. G. (1931): J. Physiol., 73, p. 67.
Gaunt, R., Nelson, W. O. and Loomis, E. (1938): Proc. Soc. exp. Biol. and Med., 39, p. 319. Quoted by Long *et al.* (*ibid*).
Grattan, J. F. and Jensen, H. (1940): J. biol. Chem., 135, p. 511.
Long, C. N. H., Katzin, B. and Fry, E. G. (1940): Endocrin., 26, p. 309.

EXPERIMENTAL DEMYELINATION OF THE CENTRAL NERVOUS SYSTEM

2. RESPIRATORY ENZYME SYSTEMS OF THE BRAIN IN POISONING WITH CYANIDE AND WITH AZIDE

by R. A. WYNDHAM[1]

(From the Institute of Medical and Veterinary Science, Adelaide).

(Accepted for publication, 12th July, 1941.)

In the first paper of this series Hurst (1940) showed that, with or without necrotic lesions in the grey or white matter, areas of demyelination may occur in the white matter of the brains of monkeys poisoned by repeated doses of potassium cyanide. Because of the rapid detoxication of cyanide in the animal body, he inferred that cyanide does not act cumulatively in the sense that its concentration steadily rises to a level injurious or fatal to the animal, but rather that each dose leaves its trace of damage and that the final result is cerebral demyelination.

The lethal effect of cyanide is usually assumed to be due to its inhibition of intracellular respiratory enzymes, notably the cytochrome oxidase system, with consequent (cerebral) anoxia. Such *reversible* inhibition is sufficient to account for the unconsciousness sustained by the animals for some minutes or hours after each dose. The ultimate death of the animals in these studies cannot always be ascribed simply to anoxia, but to causes which may or may not be entirely connected with irreparable damage to the brain.

It was decided to investigate whether or not the activity of the oxidation-reduction systems of the brain is diminished after one or many doses of potassium cyanide. This investigation was extended, in the case of monkeys, to treatment with sodium azide which, like cyanide, inhibits iron-containing respiratory catalysts. Cytochrome oxidase, catalase and the succinic acid oxidation system were studied. The succinic acid dehydrogenase has apparently a cyanide-sensitivity of its own apart from its partial dependence upon the cytochrome oxidase system in tissue-respiration (Banga *et. al.*, 1931).

EXPERIMENTAL.

In the experiments with rabbits, since it was not practicable to separate grey and white matter, the mixed tissues of the cerebral hemispheres were studied. With monkeys the grey and white matter are fairly completely separable and were studied individually. The brains were removed as rapidly as possible after death and enzyme activities determined under conditions as nearly as possible identical for any specific determination.

1. Rabbits.

Brain tissue was obtained from normal animals killed by air-embolism. For the removal of natural substrates the method used by Long and Peters (1939) was modified as follows:

Approximately 2 gm. of tissue, finely minced with stainless steel scissors, were suspended in 5 ml. of Ringer-phosphate solution (3 volumes of normal Ringer plus 1 volume of M/10 phosphate buffer of pH 7·3) and allowed to stand for 10 minutes in the cold-room at 4° C. before centrifuging. The tissue was then washed once with Ringer-phosphate, twice with 0·3 p.c. KCl solution and again twice with Ringer-phosphate, standing each time for 10 minutes in the cold-room; finally it was suspended in 5 ml. of Ringer-phosphate (pH 7·3) and strained through muslin. Of the resulting homogeneous suspension 1 ml. was taken for determination of dry weight.

Oxygen-uptake in presence of succinate. From measurements of the decoloration of methylene blue, in Thunberg tubes, with various hydrogen donators, and of oxygen-uptake with the same substrates, it was concluded that the catalytic system necessary for the oxidation of succinate was more completely represented in the tissue preparation than was that necessary for the oxidation of glucose or of glycerophosphate. The first experiments were confined, therefore, to the oxidation of succinate. The washed tissue-suspension was capable of oxygen-uptake in the

[1] Working partly under a grant from the National Health and Medical Research Council, 1940.

presence of succinate, but showed no cytochrome oxidase activity when tested by the method described below for the measurement of this activity. It was assumed, therefore, that the extent to which cytochrome oxidase took part in the oxidation of succinate by this preparation was negligible. The term "succinic dehydrogenase" has been used throughout this paper for the system which activated both succinate and oxygen. Although this usage does not conform to the usual definition it was thought inadvisable, whilst the nature of the dehydrogenase is still in doubt, either to introduce a new term or to revive "succinoxidase".

The oxygen-uptake of the tissue-preparation was measured, in air, by the Warburg (direct) method, using 16 ml. conical cups with one side-bulb and a central well. The total volume of 2·7 ml. included the tissue-suspension (1 ml., or 20–30 mg. dry weight) in the main chamber, 0·1–0·2 ml. substrate solution, in the side-bulb, of strength sufficient to give the desired concentration when mixed, and 0·2 ml. of 20 p.c. KOH placed in the central well with a small strip of No. 41 Whatman filter-paper (6 × 1 cm.) which was "accordion-pleated". The volume (2·7 ml.) was completed by the addition of Ringer-phosphate to the main chamber. The thermobarometer contained 2·5 ml. Ringer-phosphate and the usual KOH-paper. After equilibration of the apparatus at 36·9 (± 0·1)° C. for 20 minutes, adjustment of the manometers and addition of the substrate from the side-bulb, readings were taken at 5-minute intervals for half-an-hour, with a speed of shaking of 116 per minute. The oxygen uptake (expressed as μl O_2 per mg. dry weight) was plotted cumulatively, the Qo_2 (μl. per mg. per hour) being derived from the initial straight portion of the curve.

In several cases the residual Qo_2 of the preparation (without addition of substrate) was determined and found to be only 0–0·3 μl. per mg. per hour. Since, owing to the limited number of Warburg vessels, this could not be determined in every case, no correction was made for this residual respiration.

The oxygen-uptake in the presence of sodium succinate was determined in the case of 14 rabbits, employing the optimal concentration of the substrate (0·02 M). Eleven of the values thus obtained lay between 4·32 and 6·86, the average of the 14 being 5·50.

Oxygen uptake was determined, in a number of cases, in the presence of varying concentrations of sodium cyanide, which inhibited respiration as shown in Table 1.

TABLE 1.

Inhibition of respiration of rabbit-brain, in presence of succinate (0·02 M), by various concentrations of NaCN.

Rabbit No.	Qo_2 without cyanide.	NaCN concentration.	Qo_2 with cyanide.	Percentage inhibition.	Average p.c. inhibition.
49	8·10	0·0001 M	7·40	8·64	8·64
51	6·31	0·005 M	1·66	73·6	
52	4·23	„	0·67	84·1	
53	3·90	„	1·14	70·7	75·5
55	6·85	„	2·11	69·2	
56	6·86	„	1·37	80·0	
46	5·42	0·01 M	0·32	94·1	
47	4·50	„	0·47	89·6	91·4
48	5·20	„	0·50	90·4	

Incubation of tissue with cyanide. Experiments were made in which the minced tissue, before washing, was incubated at 4° or at 36° C. for 30 or 60 minutes with sodium cyanide. Control tissue was incubated simultaneously without cyanide. After subsequent washing of the tissue in the usual way, which effectively removed the cyanide, oxygen-uptake determinations in the presence of succinate, with and without cyanide, were made. In no case could any destruction of the enzyme system, as a result of the incubation with cyanide, be detected. The cyanide-sensitivity of the enzyme system *in vitro* was unaltered by the previous incubation with cyanide (Table 2).

TABLE 2.

Influence of incubation of rabbit-brain tissue with NaCN on respiration in presence of succinate (0·02 M) and its inhibition by cyanide.

				After incubation with cyanide.		
No.	NaCN concentration during incubation.	Temp. °C.	Time mins.	(a) Qo_2 in absence of NaCN.	(b) Qo_2 in presence of NaCN (0·005 M).	Percentage Inhibition.
52	0·005 M	4·0	30	4·51	1·00	77·7
54	„	4·0	30	5·77	2·15	62·7
55	„	36·2	30	6·85	1·46	78·6
56	„	36·2	60	7·85	1·37	82·5
54	0·01 M	4·0	30	6·62	2·27	65·6

It was considered to be of little value to prolong the period of contact of the tissue with cyanide, as autolytic changes, especially at the higher temperature, would be likely to spoil the preparation.

Cytochrome oxidase and catalase. For determination of cytochrome oxidase and catalase the tissue was chopped with scissors and homogenized with the simple device of Potter and Elvehjem (1936), by which a fine, uniform suspension in Ringer-phosphate was obtained.

For cytochrome oxidase the standard procedure adopted was to wash the tissue once with Ringer-phosphate (10 minutes in the cold-room), with centrifugation; further washing was found to destroy the enzyme. The tissue was resuspended in Ringer-phosphate and 1 ml. aliquots taken for dry-weight determination. The method of estimation was that of Schultze (1939): in each case the Warburg cup contained 1 ml. of tissue-suspension, 0·4 ml. of semicarbazide hydrochloride (neutralized to phenolphthalein with NaOH, final concentration 0·02 M) and 0·3 ml. of cytochrome-*c* solution[2], whilst the side bulb contained 0·25 ml. of 5×10^{-4} hydroquinone solution which was tipped in after 20 minutes' equilibration. KOH-paper, prepared as previously described, was always included. Ringer-phosphate was added to make the total volume 2·7 ml. Q_{O_2} determinations at 36·9° C. were made in the case of three normal animals (Table 3, footnote).

Sodium cyanide, in 0·005 M concentration, inhibited cytochrome oxidase activity 73·5 p.c.; in 0·0001 M concentration, 29·6 p.c.

Owing to the sensitiveness of the enzyme to washing, the effect of incubating the tissue with cyanide on the cytochrome oxidase activity could not be determined.

To obtain a catalase preparation free from haemoglobin and soluble substrates, the homogenized tissue was washed by the same procedure as that described for "succinic dehydrogenase" except that, for the final washing and suspension, 0·067 M phosphate buffer of pH 7·0 was used in place of Ringer-phosphate of pH 7·3. The estimations were made in a Warburg apparatus kept at room temperature (13·5° to 17·0° C.); each cup contained 1 ml. of tissue suspension plus 1·45 ml. of phosphate buffer, pH 7·0, whilst the side-bulb contained 0·25 ml. of 0·15 M hydrogen peroxide (made by diluting Merck "Perhydrol") which gave a final concentration on mixing of 0·015 M hydrogen peroxide. Equilibration was for 10 minutes and, after tilting, readings were taken at 2, 5, 7, 10 minutes and thereafter at 5-minute intervals, till the level of oxygen became constant in all manometers. The curve, obtained by plotting oxygen evolved (µl. per mg. dry weight of tissue) against time, was always straight for the first 5–10 minutes, so that the evolution of oxygen per hour at the initial rate was obtained by multiplying by 20 the 3-minute value read from the curve. Values varied considerably (Table 3, footnote). Cyanide (0·005 M) completely inhibited catalase activity.

In one case in which the brain gave a catalase value of 47·0 µl. O_2 per mg. per hr., a sample was also incubated with cyanide (0·005 M) for 30 minutes at 4° C. After washing, it was found to give exactly the same catalase value as the sample which had not been treated with cyanide. Owing to the great sensitiveness of catalase preparations to temperature, no incubation experiments at 36° C. were carried out. It was considered unlikely, however, that cyanide has any irreversible effect on brain catalase.

Cyanide poisoning of rabbits. Potassium cyanide was injected intravenously or subcutaneously into rabbits and the animals either died or were killed (by air-embolism) at times after the final injection varying from 1 minute to 11 hours, according to the dose and manner of administering the cyanide and to the individual susceptibilities of the animals. Immediately upon the death of the animals the enzyme determinations were made as in previous experiments. The relevant data and results are given in Table 3.

TABLE 3.

Enzyme activities in brains of rabbits poisoned with KCN.

Body wt., kg.	Total dose, mg./kg.	Route.	Time of death.	Cytochrome oxidase.	"Succinic dehydrogenase".	Catalase.
2·15	3·16*	Vein	21 m.	1·48	8·2	—
1·90	2·0	„	1¼ m.	3·30	5·72	16·7
2·35	2·8*	„	28 m.	—	—	15·0
2·27	7·0	Subcut.	†5 hr. 40 m.	2·34	—	—
2·35	7·0	„	†8 hr. 40 m.	—	—	41·9
2·10	12·2*	„	11 hr.	2·26	3·14	18·0
2·60	3·8*	Vein and subcut.	1 hr. 53 m.	1·08	—	—
2·30	1·74	Vein	1 m.	0·86	3·50	21·6

* Aggregate of 2 or 3 doses, given at intervals until animal showed no signs of recovery.

† Animal killed.

Typical results from normal rabbits were: Cytochrome oxidase: 3·38, 3·52, 3·04; "succinic dehydrogenase": 6·90, 4·50, 4·22; and catalase: 45·50, 27·00, 24·80.

[2] This was prepared from ox heart by the method of Keilin and Hartree (1937) and the iron value determined by the αα′-dipyridyl method; 0·3 ml. contained 4·8 µ Fe.

2. Monkeys.

The methods used for studying monkey-brain were the same as those used for rabbit-brain except that, for the determinations of cytochrome oxidase and catalase, the tissue was not homogenized by the technique formerly employed but was minced with scissors and the final suspension strained through muslin as in the case of "succinic dehydrogenase" determinations. This gave a much finer and more uniform preparation.

No experiments involving incubation of the tissue with cyanide *in vitro* were performed, more attention being paid to the procuring of chronic cyanide-poisoning in the animals themselves.

Normal monkeys. Four monkeys (*M. mulatta*) fasted overnight, were killed by bleeding, the brain removed and about 5 gm. each of grey and of white matter were dissected from the cerebrum. Cytochrome oxidase, catalase and, in three cases, "succinic dehydrogenase" were estimated in both tissues. The results are given in Table 4.

TABLE 4.

Enzyme activities of grey and white matter of normal monkey-brain. All values are in μl. O_2 per mg. dry weight per hour.

	Cytochrome oxidase.			"Succinic dehydrogenase."			Catalase.	
No.	Grey matter.	White matter.	White matter. / Grey matter.	Grey matter.	White matter.	White matter / Grey matter.	Grey matter.	White matter.
1	3·36	1·14	0·34	—	—	—	24·4	20·6
2	1·66	0·42	0·25	1·64	0	0	28·6	42·0
3	2·68	0·62	0·23	5·78	1·14	0·20	40·0	30·2
4	2·02	0·78	0·39	6·38	1·76	0·28	14·8	10·2

It is evident that respiration in white matter is considerably less than in grey, the difference being greater in the "succinic dehydrogenase" than in the cytochrome oxidase figures.

Catalase activity, however, is of the same order in both tissues.

Cyanide poisoning in monkeys. A few animals were poisoned with potassium cyanide by giving, intramuscularly, doses large enough to kill them after a short period of unconsciousness. These doses were determined as follows: The animals were first weighed, in the morning, under ether anaesthesia and fasted on the succeeding night; on consecutive days each animal was given, fasting, a dose of potassium cyanide which was gradually increased until, on the last occasion, the animal no longer recovered consciousness. This minimal lethal dose was 3–4 mg. per kg. In one case a greater dose (4·5 mg. per kg.) was inadvertently given with a promptly fatal result.

TABLE 5.

Intoxication of monkeys by potassium cyanide and by sodium azide.

No.	Initial weight kg.	Survival after last dose.	No. of doses.	Survival after first dose.	Range of doses mg./kg.	Total dose mg./kg.	Cytochrome oxidase. Grey.	Cytochrome oxidase. White.	"Succinic dehydrogenase." Grey.	"Succinic dehydrogenase." White.	Catalase. Grey.	Catalase. White.
Cyanide.												
5	2·50	20 mins.	1	20 mins.	—	4·5	2·44	0·36	3·12	1·12	30·2	24·6
7	1·80	*12 hr. 48 m.	2	4 days	3·0–3·5	6·5	1·68	0·44	—	—	—	—
9	2·60	2 hr. 7 m.	4	7 „	2·3–4·0	12·8	1·66	0·66	—	—	16·0	11·6
926	2·90	30 mins.	58	69 „	3·0–4·75	231	†1·22	0·62	5·14	1·15	30·6	28·0
902	2·70	24 hours	63	79 „	3·5–7·0	306	†1·14	0·48	1·54	0·86	22·0	14·2
925	4·00	1 hour	69	82 „	3·0–5·25	292	††1·14	0·74	6·04	1·98	—	—
897	4·30	2 hours	90	106 „	3·0–5·25	423	‡0·42	1·08	4·08	1·90	—	—
928	3·70	3 days	153	185 „	2·75–5·75	750	‡‡0·44	0·44	3·48	1·32	—	—
Azide.												
924	1·78	30 mins.	2	2 days	15–20	35	1·32	0·70	2·64	1·22	31·8	28·8
923	2·55	40 „	5	5 „	10–12·5	60	1·20	0·0	—	—	16·0	20·4
932	2·65	40 „	5	6 „	11–12	59	1·84	0·76	3·86	1·46	—	—
906	3·30	30 „	10	11 „	8–11	104	1·56	0·60	2·98	1·08	—	—
919	2·95	24 hours	11	11 „	5–8	84	1·16	1·16	2·64	0·84	—	—
933	2·60	50 mins.	22	25 „	6–12	176	1·32	1·08	1·68	0·60	—	—
912	2·85	25 „	33	38 „	6–10	233	1·42	0·64	3·78	1·56	—	—
931	3·65	24 hours	39	43 „	6–7	263	0·56	0·56	3·58	0·94	—	—
899	3·90	24 „	58	70 „	6–12	553	0·68	1·00	6·44	2·66	—	—
938	3·45	1 „	92	99 „	5–5·5	470	0·58	0·22	—	—	—	—

Demyelination in cerebral hemispheres: † early partial; †† moderate partial; ‡ marked partial; ‡‡ massive partial.

* Animal killed.

Further animals were poisoned by daily injection of sublethal doses of cyanide. This treatment was undertaken by Dr. Hurst and his colleagues, who also made histological examination of one half of each brain, whilst the enzyme determinations were done on the other half.

The results of all these experiments are collected in Table 5, in which the animals appear in order of increasing time of survival after the first injection.

Azide poisoning in monkeys. Since the action of sodium azide on respiratory enzyme systems is similar to that of cyanide, experiments were made using azide injections instead of cyanide. The results are recorded in Table 5.

DISCUSSION.

No satisfactory explanation can be offered for the great variation of the Qo_2 value for succinate oxidation in rabbit-brain. This value bore no relation to the time elapsing between death of the animals and the taking of measurements. Possibly the state of nutrition of the brain cells at the time of death may have had an influence; however, the "residual respiration" was just as insignificant in brains showing high, as in those showing low "succinic dehydrogenase" activity.

It is doubtful whether any of the values obtained from cyanide-poisoned rabbits are significantly lower than those from normal animals, and there is no correlation between variations in the enzyme values and variations in the duration of the exposure to cyanide. It is unlikely that cyanide, in rapidly fatal doses, produces any irreversible effect on the enzyme systems under discussion.

The average minimal lethal dose of cyanide (Table 3) is 5 mg. per kg. If the blood represents approximately one-twelfth of the animal's body-weight and this dose of cyanide becomes uniformly distributed throughout the circulation, then the concentration of cyanide which reaches the brain as a whole cannot exceed 6 mg. per litre, and is probably lower. This concentration of cyanide is of the order of 0·0001M, in which concentration cyanide *in vitro* inhibits cytochrome oxidase only 30 p.c. and "succinic dehydrogenase" only 9 p.c. It has also been shown that fifty times this concentration of cyanide has, *in vitro*, no *irreversible* action upon "succinic dehydrogenase" when in contact with the tissue for one hour. Therefore, if inhibition of these enzyme systems is the cause of death in the animals, we must draw one of the following conclusions. (1) A small impairment of cellular respiration throughout the whole brain is sufficient to cause death in a short time. (2) Certain regions of the brain are more sensitive to inhibition by cyanide than the brain as a whole and partial or complete inhibition in these regions is immediately fatal. (3) Cyanide accumulates in some cells where respiration is ultimately abolished with consequent death of the animal. It is not impossible that a portion of the cyanide escapes elimination and remains bound in some cells in a form in which its action is more gradual.

Monkeys repeatedly recover consciousness and are apparently quite normal after cyanide injections until their resistance is so lowered that they are killed by a dose which would not at first have proved fatal. This supports the third of the above hypotheses rather than the others.

On the other hand, death in monkeys may not result directly from asphyxiation of the tissues, but because permanent damage to the neural structure gradually increases during successive periods of temporary oxygen-deprivation. Demyelination might well be a manifestation of this type of process.

Various authors have observed the oxygen uptake, cytochrome–*c* and cytochrome oxidase contents of the grey and white matter of the brain (Vernon, 1911; Holmes, 1930, 1932; Krebs and Rosenhagen, 1931). The measurements of enzyme activity reported here for normal monkey brain fall in line with the earlier statements that white matter is about one-sixth to one-third as actively respiring as grey matter.

In the experiments on cyanide- and azide-poisoning, the cytochrome oxidase activity of the brain appears not to have been seriously impaired except in one case of cyanide and three cases of azide poisoning, in which the time of survival of the animals was longest and the total intake of the substances greatest. Here, however, only the grey matter showed any marked diminution of activity compared with normal.

In the case of succinate oxidation the ratio of white to grey is generally one-third or higher and in both tissues the system has resisted intensive treatment with cyanide or azide.

Catalase values, also, show no impairment even after 79 days' treatment with cyanide.

It is suggested that pure myelin may not contain any respiratory enzymes and that the low respiratory activities reported here and elsewhere may be due merely to the cells and cell-processes which form a relatively smaller proportion of the white matter than of the grey. Marinesco (1924) has observed that the dendrites can be followed throughout their course in the white matter by means of the staining of their oxidase granules with neutral red and methylene blue.

The histological results show that demyelination in the cerebral hemispheres of animals poisoned with cyanide was more pronounced in those animals which survived longer and received more cyanide. In the severest case of demyelination (928) the grey matter showed no lesions, whereas there is an apparent diminution in cytochrome oxidase activity to a greater extent in the grey matter than in the white. Hence the activities of the enzymes here reported would seem to bear no relation to the severity of demyelination.

The animals poisoned with azide consistently showed no demyelination in the cerebral hemispheres, but in other regions changes which will be described in a paper shortly to be published by Hurst. These changes can have no relation to the activities reported in the present paper.

It is concluded that the cytochrome oxidase, "succinic dehydrogenase" and catalase systems of the cerebral hemispheres are not destroyed by the poisoning with cyanide which causes demyelination in this region in monkeys, nor by poisoning with azide. There is no evidence that cerebral demyelination is due to irreversible inactivaton of the respiratory enzyme systems in the affected tissues.

SUMMARY.

Measures of "succinic dehydrogenase," cytochrome oxidase and catalase in rabbit-brain tissue have been made *in vitro*.

Incubation of the tissue with cyanide produced no irreversible effect on "succinic dehydrogenase" and cytochrome oxidase.

Tissue from rabbits poisoned with cyanide, in short-term experiments, showed no variations in enzyme activity consistent with the intensity of the dosing with cyanide.

In monkeys the same activities were measured in the grey and white matter of the normal brain and of the brains of animals chronically poisoned with cyanide and azide.

Cyanide and azide produced no change in "succinic dehydrogenase" or catalase activities, whilst cytochrome oxidase activity appeared to be diminished by prolonged treatment of the animals, but chiefly in the grey matter.

There is no evidence that demyelination in the cerebral hemispheres of monkeys is due to permanent inactivation of the respiratory enzymes.

Acknowledgments. The author wishes to thank Professors E. Weston Hurst and M. L. Mitchell, of Adelaide, and Dr. R. Lemberg, of Sydney, for their helpful criticism and advice; also Mr. H. R. Marston, of the Animal Nutrition Laboratory, Adelaide, for allowing him the use of a Warburg apparatus.

REFERENCES.

Banga, I., Schneider, L. and Szent-Györgyi, A. (1931): Biochem. Z., 240, p. 454.,
Holmes, E. G. (1930): Biochem. J., 24, p. 914.
Holmes, E. G. (1932): *Ibid.*, 26, p. 2005.
Hurst, E. W. (1940): Austral. J. exp. Biol., 18, p. 201.
Keilin, D. and Hartree, E. F. (1937): Proc. Roy. Soc. London, B. 122, p. 298.
Krebs, H. A. and Rosenhagen, H. (1931): Z. ges. Neurol. u. Psychiatr., 134, p. 643.
Long, C. and Peters, R. A. (1939): Biochem, J, 33, p. 759.
Marinesco, G. (1924): Schweiz. Arch. Neurol. u. Psychiatr., 15, p. 3.
Potter, V. R. and Elvehjem, C. A. (1936): J. biol. Chem., 114, p. 495.
Schultze, M. O. (1939): *Ibid.*, 129, p. 729.
Vernon, H. M. (1911): J. Physiol., 42, p. 402; *ibid.*, 43, p. 96.